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UNIVERSITY OF ALBERTA

STUDIES ON THE REPRESSION OF F PLASMID TRANSFER

BY

STUART LEE

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF BIOCHEMISTRY

Edmonton, Alberta
Fall, 1991

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

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June 21, 1991.

Abstract

Bacterial conjugation is a directed transfer of genetic information from a donor cell to a recipient cell and is performed by gene products encoded by conjugal plasmids, whose specific function is to transfer DNA. This process is an important one because it allows for the transfer of DNA between different bacterial species, it contributes to genetic variability and flexibility of bacteria in nature.

This thesis addresses a number of aspects concerning the control of conjugal DNA transfer in the F plasmid, a prototype conjugal plasmid. F plasimd transfer is dependent on the expression of a protein, TraJ, which activates transcription of the transfer operon, a 35 kb operon that encodes most of the gene products required for conjugation. *traJ* expression is repressed by the fertility inhibition or 'fin' system. This system consists of the products of two genes, *finP* and *finO*; both of which are necessary for transfer repression. FinP is an approximately 80 base RNA molecule that is complementary to the 5' untranslated portion of the *traJ* mRNA. FinO is most probably a protein. FinOP have been shown to operate at the transcriptional level, reducing the amount of *traJ* mRNA in cells containing the F plasmid.

The nature of FinO:FinP and FinOP:*traJ* mRNA interactions was investigated through a series of *in vivo* experiments. The *traJ*RNA:FinOP interaction was examined through the cloning and induced expression of *traJ* in the presence and absence of FinOP; the fate of the expressed *traJ* RNA was monitored through Northern blotting and probing with a probe complementray to *traJ*. This experiment suggested that FinOP may not be acting post-transcriptionally. Then, the repressive ability of either *finP* itself or its promoter was elaborated, through introducing a site-specific mutation into the F plasmid that abolished *finP* transcription, and examining the resulting mutant strains of bacteria for transfer and repression functions. Although the site-specific mutant

produced more *traJ* RNA, it did not transfer more efficiently than the wild-type F plasmid. The contribution of transcripts originating at promoters upstream of *traJ* to the control of repression was evaluated through constructing a number of different transfer region clones and assessing their affect on F-bearing cells' transfer efficiency. These experiments demonstrated that *traM* transcripts did not play a role in the transfer repression system of the F plasmid. Finally, the nature of FinOP interaction was studied through inducing *finP* expression in the presence and absence of *finO*, and noting any differences in the stability of FinP. The presence of *finO* in a cell increased the half-life of *finP* RNA from about 7.5 minutes to over forty minutes.

The results from these experiments suggested a model of FinOP action that is different from current paradigms of antisense RNA acitvity.

ACKNOWLEDGEMENTS

In the long course of studies leading to this degree, I have worked and played with a lot of people and they have all been crucial to the successful completion of my graduate career.

Lab folks - the people I've worked with in the various labs - have been a constant source of nurturing, amusement, annoyance and inspiration. I have to say that working and joking with them all and learning to accept everyone has been the thing I enjoyed the most about this place. In the early days, I did time with Gillian Shaw, Doug MacIssac, Russ Bishop, Caro Condon, Lynne Elmes, David Latour, and Damaraju Sambasivarao (Sambu). Gillian was always a source of support and dry British wit; and David, with his intellectually demanding mind and cynical, yet strangely hopeful outlook on things provided me new perspectives on scientific thought. Doug the rugger-man was a wonderful friend and all-round good guy and role model. And I'll remain eternally grateful for the two spectacular introductions to Indian cooking I received from Sambu and his wife.

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Moving right along to the sex lab where I've spent the last 11/2 years confined in a windowless room with 3 other grad students. It's been nice working with Sonya, the only Afrikaans speaking student in the department. Ken, our all-parts-complete-and-present computer gnome has been extremely patient with helping this artsie-in-a-science-world novice computer hack through the intricacies of Macintosh software. He has also proved a fun guy to do things with whether it be boot skiing in the Rockies or discussing the implications of chaos. Through the last year Tim has been my cuddle-bunny and playmate, while garishly displaying his ample ... scientific ability to everyone in the lab. Last, and the inverse of least is Dila; it has been an inspiration to watch her methodically create a life built on self-awareness, caring for others, and honest ambition in science. Her integrity and honesty make this lab a much more serene, secure environment for those of us a little less well balanced.

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Abbreviations Used in This Thesis

A	Adenine
C, CTP	Cytidine, Cytosine Triphosophate
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonulease
G	Guanine
HPLC	High Pressure Liquid Chromatography
IPTG	Isoppropyl β-D-thiogalactopyranoside
kb	kilobase (1000 bases)
kd	kilodalton (1000 daltons)
OD	Optical Density
ORF	Open Reading Frame
P	promoter
PAG, PAGE	Polyacrylamide Gel, Polyacrylamide Gel Electrophoresis
POPOP	1,4-bis[5-Phenyl-2-oxazolyl]benzene;2,2'-p-phenylyene-bis[5-phenyl oxazole]
PPO	2,5 diphenyloxazole
prPa	oligonucleotide primer complementary to FinP
prJ1	oligonucleotide primer complementary to traJ RNA
RNA	Ribonucleic Acid
mRNA	messenger Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
tRNA	transfer Ribonucleic Acid
RNase	Ribonuclease
SDS	Sodium Dodecyl Sulfate
T	Thymine
'X'	Fold, ie 24X=24 - fold,
Xgal	4-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
φ	bacteriophage

In the text of the thesis, other than strain genotypes, an italicized gene locus name indicates the wild-type allele of the gene.i.e.*finP*

In the text of the thesis, other than strain genotypes, a mutant allele of a gene is indicated with a "-". i.e. *finP-*

When used in a description of a strain genotype, an italicized gene locus name refers to a mutation in that gene. i.e.*trp*

The normal print gene locus name refers to the gene product. i.e. FinP is the gene product of *finP*.

Chapter I. Introduction

A. Background Concepts

Bacterial conjugation is the directed transfer of genetic information from a donor cell to a recipient cell and is performed by gene products whose specific function is to transfer DNA. The gene products are encoded by conjugal plasmids, and transfer the plasmid which encodes them. This process is an important one, on many different levels. Because it allows for the transfer of DNA between different bacterial species, it contributes to genetic variability and flexibility of bacteria in nature. Bacteria in the wild have, in effect, access to an expanded genome, for often the plasmids contain parts of bacterial chromosomes and/or functions specific to these plasmids. Of interest to man is the rapid spread of antibiotic resistance among bacterial pathogens - a conjugal plasmid-mediated event. The ability for resistance to environmental toxins such as heavy metals is often carried on conjugal plasmids. Also, the introduction of genes onto conjugal plasmids whose products degrade pollutants carries with it the hope that these abilities will spread through a population of bacteria living in a polluted environment. This process of directed, specific information transfer between bacteria, then, is clearly an important process in nature for the dissemination of information and function.

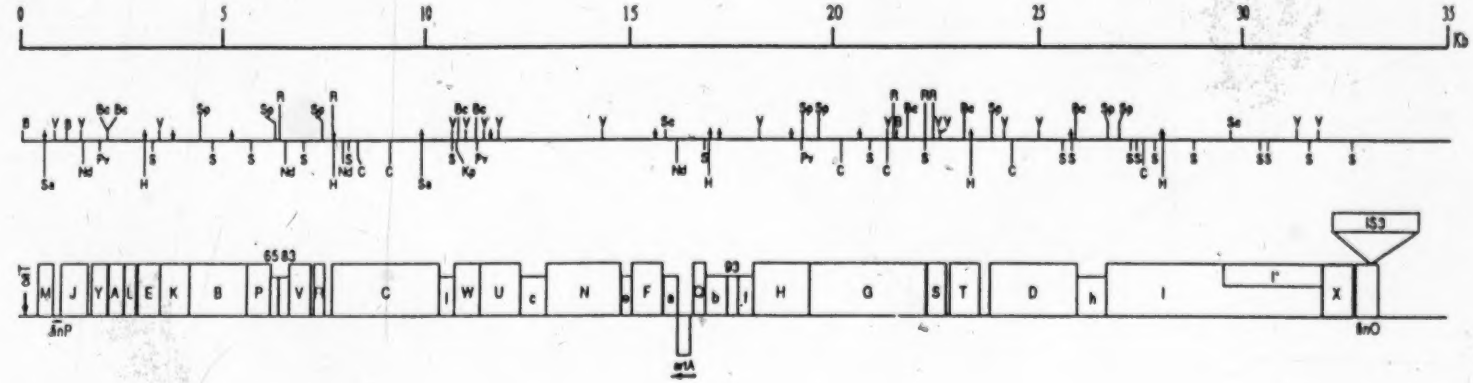
The best characterized conjugal plasmid is the F plasmid. It was discovered in the 1940's in Joshua Lederberg's laboratory (Cavalli-Sforza *et al.*, 1953; Hayes, 1953). In that case, the F plasmid turned out to be a cointegrate in the *E. coli* chromosome (Cavalli-

Sforza *et al.*, 1953; Hayes, 1953). Normally, though, the F plasmid is a DNA molecule of 100kb that exists in the cell independent of the chromosome. Through the history of molecular biology, it has served a number of important functions. Some of these are related to its transfer ability, such as being a necessary co-resident in cells used to grow phage for M13 sequencing (Yanisch-Perron *et al.*, 1985). It is also used as a vehicle for the introduction of complementing genes in merozygote strains of *E. coli* and the delineation of *cis* versus *trans* effects of mutations and/or gene products. Such a ubiquitous lab tool seems, therefore, the natural choice for genetic studies. The studies on F plasmid transfer began in 1969 in the laboratory of John Ciark .

These and subsequent studies in other laboratories resulted in the sequencing of the entire transfer region of the plasmid, 35 kb of DNA, and biochemical characterization of many of the participatory proteins (Willetts, Skurray, 1987). Although this accumulation of knowledge is substantial, the actual mechanism of gene transfer during plasmid conjugation remains unclear. But some of the basic steps of conjugative transfer have been inferred from the genetic and biochemical studies undertaken.

The F plasmid is 100 kb long, and exists in the bacterial cell as a closed circle of DNA; it is closely associated with both the cellular membrane and the chromosome (Willetts, Skurray, 1987). Almost exactly one-third of the plasmid's sequence is devoted to encoding transfer functions. The 35 kb that make up the transfer operon lie in a contiguous arrangement, and include about 24 identified genes along with about 10 more open reading frames (ORF's) whose functions remain unknown (Fig. I.1) (Willetts, Skurray, 1987). The expression of most of these genes is dependent on the activity of the promoter preceding *traY*, P_Y, which in turn is dependent on the positive regulator TraJ (Mullineaux, Willetts, 1985), a protein encoded by the gene that lies immediately upstream of *traY* (Helmuth, Achtman, 1975; Bradley, Coetzee, 1982). Once transcription begins at P_Y, it is thought that it continues through the rest of the transfer region,

Figure I.1 The genetic map of the F plasmid transfer region. Transcription of most of the operon begins at *traY* and continues rightward. *traM* and *traJ* have promoters that direct transcription in the same direction as P_Y. *finP* and *artA* have promoters that direct transcription in the opposite direction. There are other supplementry promoters within the operon that promote transcription in the same direction as P_Y. Gene products whose functions are known to be related are grouped together in the lower part of the figure. This figure is from Dr. Karin Ippen-Ihler, Texas A&M University.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X
IS3
oriV
orf4
Pilus synthesis and assembly
Surface exclusion
Aggregate stability
Surface exclusion
Signal, origin nicking, unwinding, and transport

producing a very long polycistronic mRNA (Willetts, Skurray, 1987). The gene products encoded by this transcript participate in the processing and transport of pilin to the outer membrane (Willetts, Skurray, 1987). Pilin monomers are then assembled into long proteinaceous structures called pili (or fimbriae) that project out from the cell (Bradley *et al.*, 1980). The pili bind to receptors on the recipient cell outer membrane, (Willetts, Skurray, 1980) and it is assumed that they retract back into the donor cell, pulling the recipient along (Achtman *et al.*, 1977; Novotny, Fives-Taylor, 1974). After cell:cell contact is initiated, it is hypothesized that a triggering event occurs, and the plasmid is nicked at the origin of transfer, *oriT* (Willetts, Skurray, 1987). The gene products TraY, TraI, and TraM, are bound to *oriT* in a massive complex, involving upwards of fifty copies of TraI (Abdel-Monem *et al.*, 1983), and an unspecified but numerous number of TraM molecules (L. DiLaurenzio, pers. comm.) and a binding site for TraY (Lahue, Matson, 1990). TraY nicks (Willetts, Skurray, 1987), TraM binds to (L. DiLaurenzio, pers. comm.), and TraI (DNA helicase I) unwinds the F plasmid (Abdel-Monem *et al.*, 1983) to perform the process of conjugation. A single strand of F plasmid DNA is transported first to the periplasm of the donor, then to the recipient cell, where it is replicated as it enters (M. Durrenberger, unpubl. res.). The F plasmid also contains two genes, *traS* and *traT*, whose gene products ensure that once mated into a cell, there will be no further F introduction into that cell (Achtman *et al.*, 1977; Achtman *et al.*, 1980). The foregoing is a basic overview of the process of F-mediated DNA transfer, as it is understood today.

The idea of transfer repression came from studies involving plasmids related to F. On the basis of a number of classification schemes, including pilus morphology (phage sensitivity), transfer gene complementation, DNA hybridization, size, and gene organization, there are a number of plasmids that are considered F-like in nature, and they fall into four different incompatibility groups, termed F-I to F-IV (Willetts, Maule, 1985). These plasmids, though very similar to the F plasmid, often transfer DNA at frequencies 1/100 to 1/1000 that of F itself (Finnegan, Willetts, 1971). When present in the same cell

as the F plasmid, they repress its transfer (Finnegan, Willetts, 1971). An experiment demonstrating this phenomenon is diagrammed in Figure I.2. It is to be noted that R100 is an F-like plasmid. Clearly, a gene product found on R100 was repressing both plasmids' transfer ability. The newly discovered repression system was called the fertility inhibition or 'fin' system (Finnegan, Willetts, 1973), and the newly discovered gene was called *finO*.

B. Early Genetic Investigations

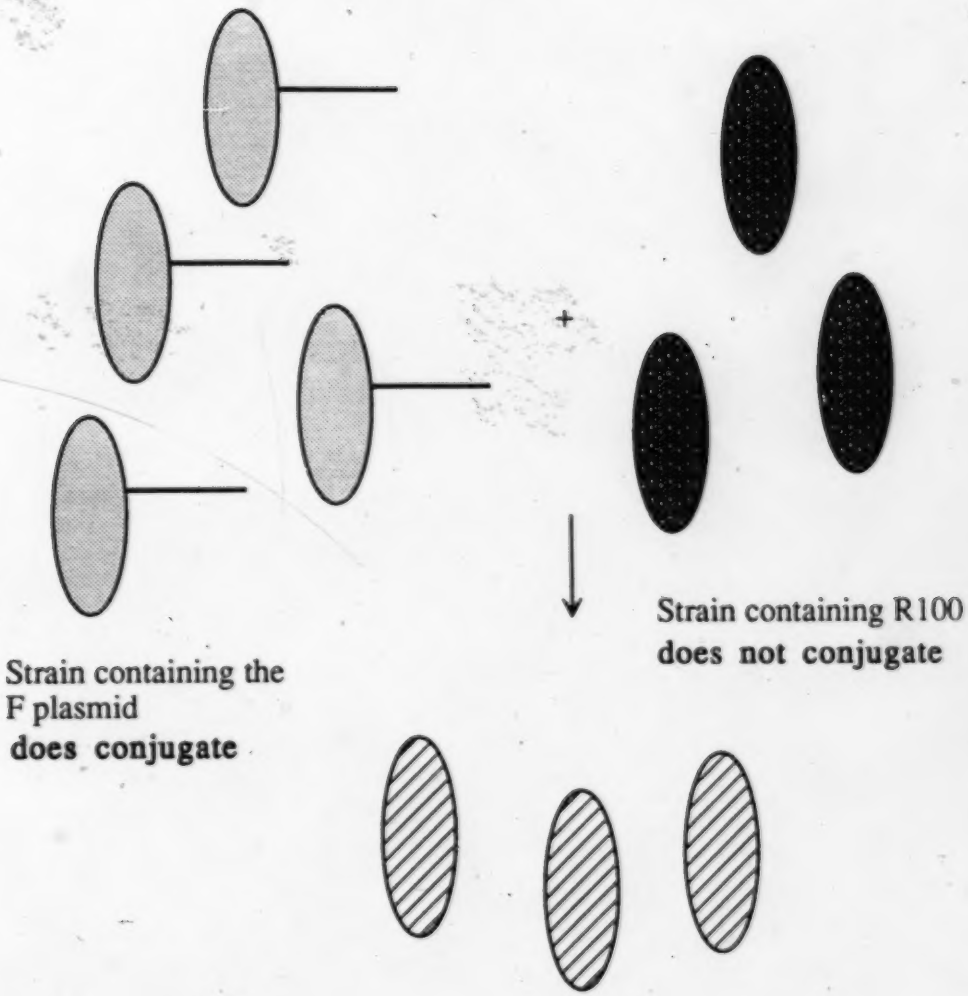
In 1971, it was observed that certain mutants of R100, an F-like plasmid, could not repress their own transfer, but still could repress F's. There had also been reports of a mutation in an F-bearing strain that relieved the R100-induced repression of F, but it was not clear whether that mutation resided on the F plasmid or not (Finnegan, Willetts, 1971). These observations suggested that more than one gene product was participating in transfer repression. Finnegan and Willetts (Finnegan, Willetts, 1971) performed a series of experiments that involved a mutational analysis of the transfer region of the F plasmid. They isolated mutants that caused the derepression of F transfer in the presence of R100. In cells containing both the mutant and a wild-type F plasmid along with R100, the inhibition of transfer was re-established (see Fig. I.3). This result implied that there was a gene product encoded by the wild-type F plasmid that interacted with the R100 FinO to inhibit transfer, and that it could function in *trans* to repress a derepressed mutant. This newly described molecule's gene was called *finP*.

Because the discovered *finP* mutation was recessive, it was complemented by the presence of a wild-type F plasmid. The researchers discovered a second class of *finP* mutation, one that was dominant because it affected the way that FinO and FinP interacted as a functional complex. This mutation was called *fisO*. In the presence of R100, a wild-type F plasmid only partially repressed the transfer of the *fisO* mutant plasmid.

Through this mutational analysis, it was established that the F plasmid's ability to conjugally transfer itself was repressible through an interaction of a 'broad host-range'

Figure I.2 A schematic diagram of the experiment used to discover the phenomenom of fertility inhibition. The ovals represent individual bacteria, the different shading indicates different bacterial strains, and the little rods extending from the ovals represent pili. All bacteria capable of conjugation have pili; those that are not capable of conjugation have no pili.

Discovery of *finO*



Strain containing the
F plasmid
does conjugate

Strain containing R100
does not conjugate

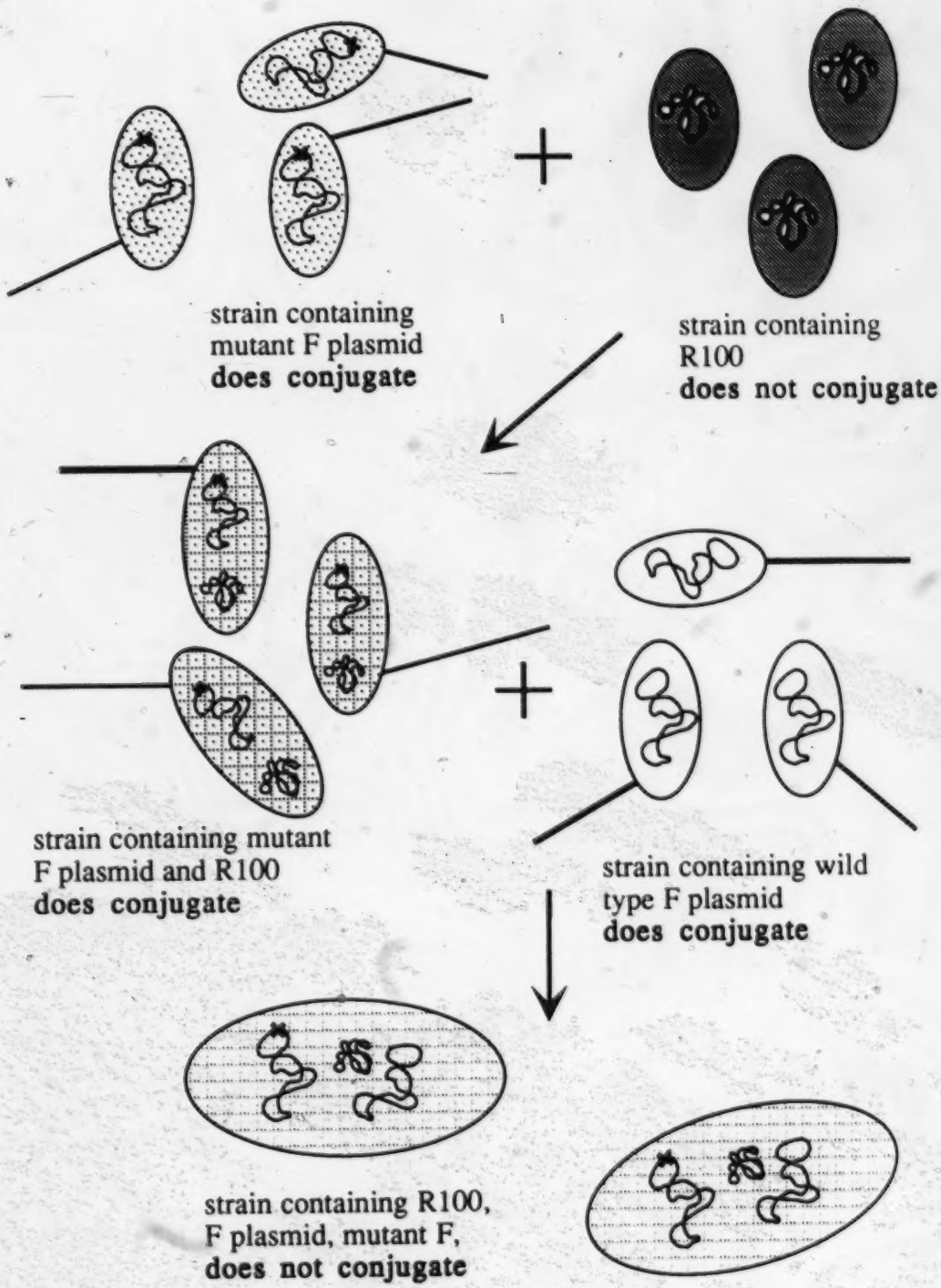
Strain containing F plasmid
and R100
does not conjugate

Conclusion:

A gene product supplied by R100 repressed a transfer activating function encoded by the F plasmid

Figure I.3 A diagram of the experiment used to discover *finP*. The ovals represent individual bacteria, the different shading indicates different bacterial strains, and the little rods extending from the ovals represent pili. All bacteria capable of conjugation have pili; those that are not capable of conjugation have no pili. The curved lines inside the bacteria represent conjugal plasmids. The long extended one is the F plasmid, while the more compact one is R100. An 'X' on the plasmid represents a mutation.

Discovery of *finP*



Conclusion:
A gene product from F as well as R100 is necessary for repression of F plasmid transfer

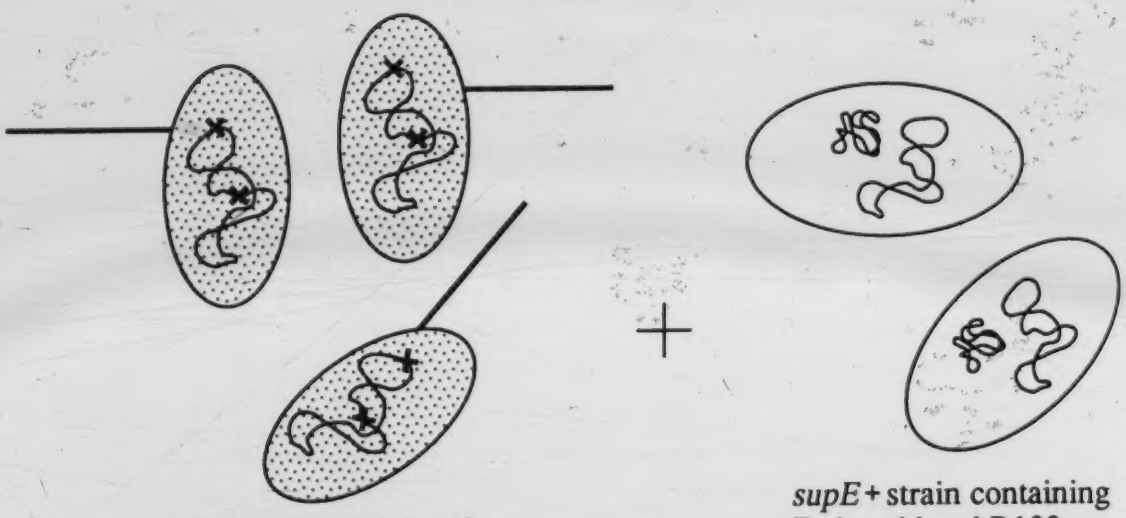
repressor supplied *in trans* by other F-like plasmids, called FinO, and a second, plasmid-specific one that it produced, called FinP (Finnegan, Willetts, 1971). This repression 'team' was termed FinOP. Neither component alone was able to repress F plasmid transfer.

Next, the target of the *fin* system was determined by a set of experiments diagrammed in Fig.I.4 (Finnegan, Willetts, 1973). Since the *fin* system acted to negate an otherwise expressed function (i.e., transfer), it was hypothesized that FinOP acted to repress the activity of a gene or gene product that activated transfer. The gene encoding the activator was determined through experiments that measured reciprocal complementation between two F plasmids, one that was wild type and repressed, and one that was derepressed and mutant in a transfer operon gene.

A series of double mutant F plasmids were constructed. Each mutant plasmid had the *fisO* mutation, which allowed the plasmid to escape *fin* repression. And each mutant carried an amber mutation in one of the 11 identified transfer genes. This derepressed double mutant F plasmid was mated out of a supressor strain, where the transfer operon mutation was compensated for, into a strain of bacteria with a wild-type F plasmid repressed by a co-resident R100. Since the mutant plasmid was *fisO*, and therefore unrepressible, it could serve as a source of the activator of the transfer operon. The wild-type F plasmid served as a potential source of wild-type transfer gene products, providing its transfer operon was activated. As long as the derepressed mutant was not mutated for its activator function, it would produce the activator molecule, and complement the repressed wild-type plasmid, thus enabling the expression of the wild-type transfer operon. This expression would result in the production of the wild-type transfer gene product that the mutant was lacking. Then the mutant would be able to transfer out of the intermediate strain. But if the transfer operon mutation was in the gene for the activator molecule, then the mutant F plasmid would not be able to complement the wild-type F plasmid, which, in turn, could not complement the mutant, and both plasmids would remain in the intermediate

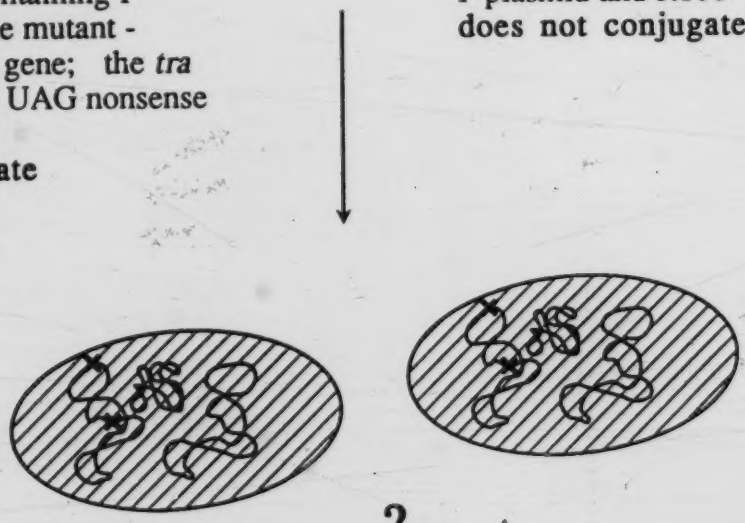
Figure I.4 A diagram of the experiments used to determine the target of FinOP.The ovals represent individual bacteria, the different shading indicates different bacterial strains, and the little rods extending from the ovals represent pili. All bacteria capable of conjugation have pili; those that are not capable of conjugation have no pili. The curved lines inside the bacteria represent conjugal plasmids. The long extended one is the F plasmid, while the more compact one is R100. An 'X' on the plasmid represents a mutation. *supE* refers to a mutation in the host strain that fails to recognize the UAG (amber) stop codon. Therefore in this strain a gene containing a premature termination codon (amber mutation) will be expressed. A *supE*$^+$ strain does not have this mutation.

Experiment to Determine the Activator of the Transfer Operon and the Target of the *fin* System



supE strain containing F plasmid double mutant - *finP* and a *tra* gene; the *tra* mutation is an UAG nonsense mutation
does conjugate

supE+ strain containing F plasmid and R100
does not conjugate



?

supE+strain containing mutant and wild-type F plasmid, and R100. Can conjugate only if mutant can supply *tra* operon activator to the wild-type F plasmid.

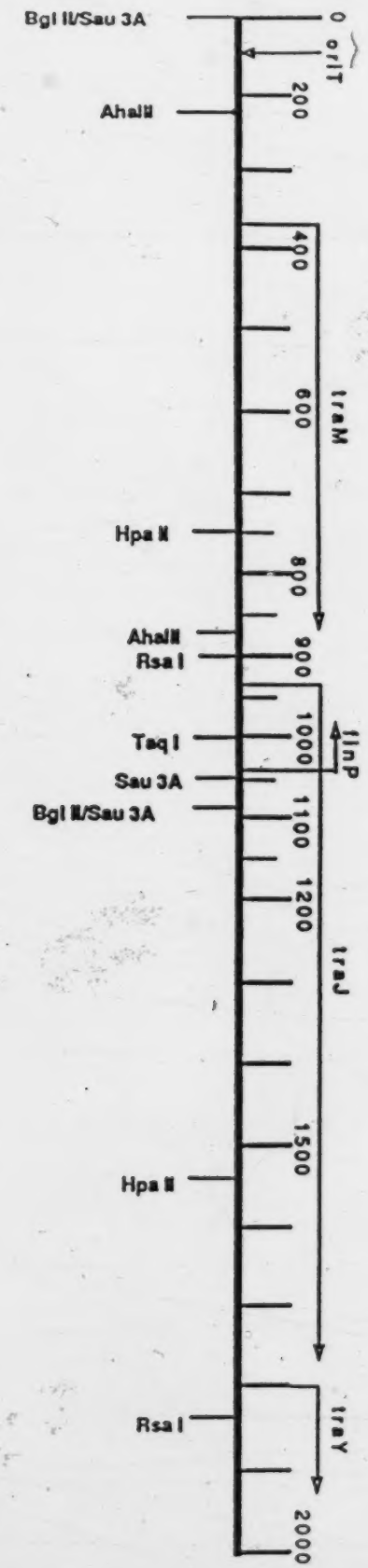
strain. The assay measured the frequency of mutant plasmid transfer out of the intermediate strain. These experiments showed that the gene *traJ* was the 'target' of the fertility inhibition system of F-like plasmids. And since the repression of fertility resulted in a pleiotropic reduction of transfer-associated phenotypes, TraJ was given the role of a positive regulator of the transfer operon.

The next fifteen years' work was devoted to the precise mapping and sequencing of the transfer region, the identification of the transcripts in the control area of the transfer operon, and the quantification of the effect of TraJ on its target promoters, along with the effect of *finOP* on *traJ* expression. Also, the molecular basis for non-complementing transfer alleles among F-like plasmids was determined, based on differences in DNA sequence. A summary of the sequencing and transcript mapping data for the control region appears on the following page in diagrammatic form (Fig. I.5) (Willetts, Skurray, 1987).

In this early era of cloning, Willetts' lab was predominant. The direction of transcription from the promoters involved in transfer were determined by examining lambda:F_{lac} transductants on agarose denaturing gels. These gels separated the two DNA strands, then the gels were blotted to nitrocellulose, and the blots were probed with *in-vivo* labelled mRNA (Gaffney *et al.*, 1983). These blots demonstrated that transcription of *traJ* and Py proceeded in the direction indicated in the map provided. In this paper, it was also demonstrated that the FinOP system acted at the transcriptional level, reducing the amount of *traJ* mRNA, as well as the amount of detectable transfer operon mRNA. Next, the extent of the control molecules' effect on the transfer system was measured, using cloned constructs that fused the *traJ*, *traM* and *finP* promoters to *lacZ* (Gaffney *et al.*, 1983). From these experiments, it was shown that:

i) The *traJ* promoter was very strong, promoting transcription at a rate 2.4X that of the fully-induced *lac* promoter (although the *traJ* fusion construct was on a multicopy plasmid, and the *lac* promoter it was compared to was on an F-plasmid).

Figure I.5 A map of the regulatory region of the F plasmid transfer region, including strategic restriction enzyme recognition sites. The arrows represent the actual size of the transcript indicated. The numbers refer to the number of base pairs along the DNA sequence.


Bgl II/Sau 3A
0
oriT
200
AhaIII
400
traM
600
Hpa II
800
AhaIII
Rsa I
900
1000
traN
Taq I
Sau 3A
Bgl II/Sau 3A
1100
1200
traJ
1500
Hpa II
Rsa I
traY
2000

ii) When in the presence of multicopy *finOP*, ß-galactosidase expression from the *traJ* fusion plasmid was reduced 60-fold.

iii) The presence of *traJ* enhanced expression from the *traM* promoter 9-fold.

This publication again demonstrated that FinOP repressed transcriptional activity from the *traJ* promoter. A second set of experiments involving transcriptional gene fusions was performed, this time using galactokinase activity to measure the promoter activity (Mullineaux, Willetts, 1985). The promoters for *traM*, *traJ*, *finP*, *traY*, *traT* and *traI* were tested for transcriptional activity. Galactokinase levels were measured both in the presence and absence of R100, a source of *finO*. Unlike the previous study, the *traM* promoter was not affected by TraJ. P_{traJ} activity was reduced only three-fold in the presence of *finOP*, but that was explainable in terms of multicopy effect, as *finO* was present on a single-copy plasmid and P_{traJ} was cloned on a multicopy vector. P_{traY} levels were reduced 15-fold in the presence of *finOP*, and P_{traM} $P_{traI}$and P_{finP} were not affected by FinOP's presence.

These studies, when combined with data derived from transcript mapping experiments (Thompsom, Taylor, 1982; Fowler, Thompson, 1986; Fowler *et al.*, 1983), gave a comprehensive picture of the overall nature of the regulation of transfer of the F plasmid. The FinOP complex reduced the amount of *traJ* mRNA in the cell, and this reduction, along with the concomitant reduction of TraJ, lowered the activity of the *traY* promoter, which was responsible for the transcription of the majority of the transfer genes. The challenge in the late '80's was the description of the FinOP complex, and the elucidation of its mechanism of action.

C. The Nature of FinO

The exact identity of the molecule that is resopnsible for *finO* activity is still unknown. Much of the early research on the molecule yielded inconsistent results. The following section of the introduction is meant to communicate the sense of confusion that surrounded the study of FinO. The most likely *finO* product is a protein. The cause of

finO activity was first investigated by Timmis *et al.* in Achtman's laboratory (Timmis *et al.*, 1978). *Eco*R1 and *Hind*III digests of the *finO*+ plasmid R6-5 were cloned into multicopy vectors. These cloned fragments were transformed into cells containing R100-1, a *finO* mutant of the plasmid R100, and their *finO* activity was measured by their ability to protect the cells against pilus-specific bacteriophage (FinOP-mediated repression of the transfer operon inhibits the production of pili, therefore the cells are immune to the phages). Through these studies, the location of *finO* was narrowed down to a 4.5 kb *Pst*I fragment. This fragment was cloned into pBR322, and then introduced into minicells. *finO*-containing minicells were incubated in the presence of ^{14}C amino acids, and the resulting proteins were analyzed by PAGE. In all the constructs containing *finO* activity, but not in those without it, a 20kd protein was usually seen that was not detected in minicells containing only vector. But since its appearance was variable, and it migrated very close to a vector-encoded protein, the identification of this gene product as FinO remained tentative. In 1984, Cheah and Skurray also cloned *finO*, using Tn5 mutagenesis to narrow the gene to a 0.5 kb region of R6-5 DNA (Cheah *et al.*, 1984). They were unable to detect any protein in ^{35}S labelled minicells, regardless of the vector they used. In a recent publication, submitted to Plasmid by Skurray et al., the region around *finO* was sequenced, and an ORF capable of coding for a 22kd protein was discovered in the area that had been mapped as *finO*.

The research on the *finO* gene of R100 was a little more thorough, yet not any more enlightening. The gene was originally cloned using lambda cointegrates that expressed *finO* activity, this time in terms of repressing co-resident F plasmids' ability to transfer (Dempsey, McIntire, 1983). Once an appropriate *finO* phage was discovered, it was tested for its ability to make a unique protein of about 20kd. No such protein was found. In fact, no protein was discovered that could be related to *finO* activity. When the phages were induced to proliferate with ultraviolet light, however, a 21kd protein was observed in phages that contained *finO* activity. But, again, its molecular weight was very similar to a

phage-encoded gene product whose expression was variable. Once again, the identification of a 21kd gene product as the cause of FinO activity was 'tentative'. The sequence of the *finO* gene of R100 was published within the same year by two laboratories, Dempsey's (McIntire, Dempsey, 1987) and Ohtsubo's (Yoshioka *et al.*, 1987). Both reported an ORF capable of coding for a very hydrophilic protein of 21.5 kd, with about 20% basic residues. Dempsey expressed this protein using an IPTG-inducible *tac* promoter (McIntire, Dempsey, 1987). The plasmid proved very unstable. Nonetheless, a protein of 20.6 kd was detected in extracts of induced cells, but its identification as the true FinO remained tentative, because Dempsey noted that in cultures carrying the construct along with an F plasmid, phage resistance was more likely to be conferred by curing the cells of the F plasmid. Thus the observation of the 20.6 kd protein may not have had anything to do with *finO* activity. Mutations were made in the ORF, in hopes of demonstrating a definitive phenotype. The fragment containing the ORF was digested internally at a single site with *Xma*I, then treated with the Klenow fragment of DNA polymerase I, which had the ability to 'chew back' the DNA strand it was processing. In this manner, a number of internal deletions of the *finO* ORF were created. Again the results were equivocal. Two of the mutant constructs would produce truncated proteins as a result of a frameshift induced by the mutation event. But the N-terminal 49 amino acids, rich in basic residues, were maintained. One of these mutants, only 63 residues long, had almost wild-type activity; the other also repressed F transfer, but an order of magnitude less efficiently. Two other deletions that deleted beyond amino acid 49, however, could repress very poorly - 10-30-fold worse than the previously mentioned mutant. The protein could not tolerate deletions in the N-terminus - the removal of the first 5 amino acids resulted in an abolition of *finO* activity. A mutation that fused the C-terminus of the putative *finO* ORF to pBR322 sequences also had no activity. These difficult-to-interpret results, coupled with the inconsistent appearance of the protein, led Dempsey and McIntyre to suggest that perhaps

RNA transcripts originating from the 'non-coding' strand were the real executors of *finO* activity (McIntire, Dempsey, 1987).

Yoshioka et al. presented a much simpler analysis of the *finO* gene (Yoshioka *et al.*, 1987). They cloned the gene and sequenced it, and expressed a 22kd protein product in maxicells. This protein product was not present when a clone containing a truncated *finO* ORF was used in the maxicell experiment. To support their contention that the 22kd protein was the authentic FinO, they sequenced the *finO* gene of the *finO* mutant of R100, R100-1, and discovered that this mutation was due to the insertion of an adenine residue at approximately amino acid 50, and results in the premature termination of the gene at approximately amino acid 70. The sequence of F in the region of *finO* was also reported, and it was discovered that F contained a *finO* gene very similar to that of R100 and R6-5, but an IS3 insertion element had inserted itself into the F coding sequence. These workers therefore claimed that *finO* activity is effected by a protein.

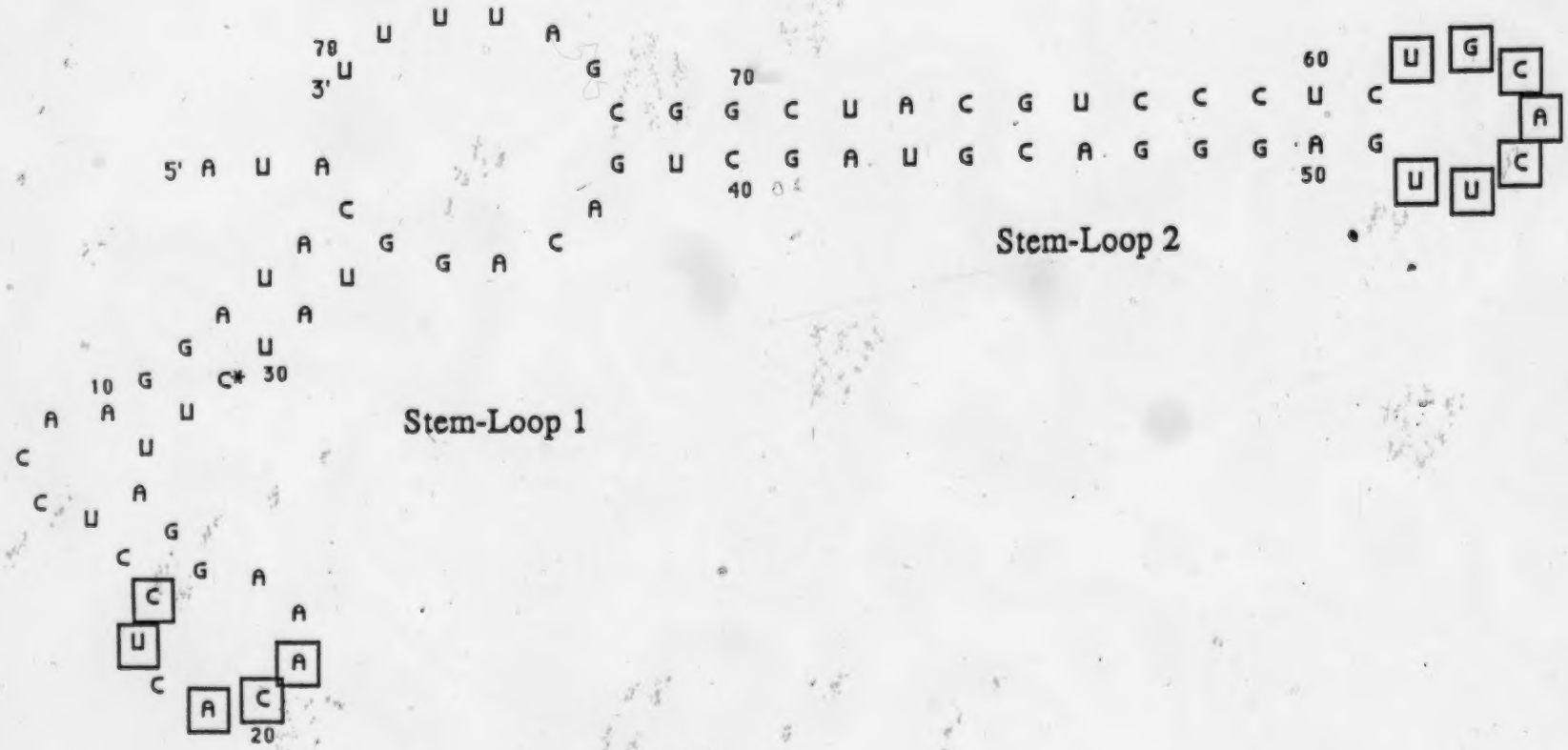
Recent unpublished results from the laboratory of Frost in the Department of Microbiology, Universtiy of Alberta indicate that a 22kd protein can consistently be expressed from a clone containing the *finO* gene (T. van Beisen, pers. comm.). Although these results and the ones reported by Yoshioka et al. suggest FinO is a protein, there is uncertainty about its true nature. This uncertainty exists because no one has been able to isolate or detect FinO from cells containing F-like plasmids, its mRNA has never been detected, and a promoter for *finO* has not been detected through sequence analysis. On the other hand, Dempsey (Dempsey, 1987) has reported detecting RNA transcripts originating from the DNA strand complementary to the proposed *finO* ORF. Until someone unequivocally shows a FinO protein product originating from an F-like plasmid, the F plasmid community will be unsure of the nature of FinO. For the purposes of this thesis, FinO is considered to be a protein, with the caveat that it may be something else. Some of the experiments performed will address the nature of its interaction with FinP and the interpretations of these experimental results will address this ambiguity.

D. The Nature of FinP

The first proposal that FinP may be an RNA transcript complementary to the 5' 150 nucleotides of *traJ* appeared in a paper by Mullineaux and Willetts (Mullineaux, Willetts, 1985). They localized *finP* function to an 80 base-pair *Bgl*II-*Taq*I fragment, then searched the sequence of the fragment for promoters and found P_{finP}, which would promote transcription from the coding strand of the *traJ* gene, producing a transcript complementarty to the *traJ* message. They proposed that the executor of *finP* activity was an antisense RNA molecule. Finlay et al. (Finlay *et al.*, 1986) addressed this proposition by sequencing all the F-like plasmid alleles of *finP*. They discovered that the proposed *finP* transcript could be folded into an RNA molecule consisting almost entirely of a stem-loop structure (Fig. I.6), which is a typical structure for antisense RNA molecules. All the sequence differences between the different alleles of F-like plasmids existed in the loops. This is significant because in the general paradigm of antisense RNA activity, the initial recognition interaction between the antisense RNA and its target is mediated through base-pairing between the single-stranded loop regions of the two molecules. The differences in the loops would also explain the strain-specificity of *finP*. Based on sequence similarities and thermodynamic considerations a molecular model of FinP was proposed. The diagram shown in Figure I.6 excludes the third proposed stem loop, because subsequent experiments have shown that FinP is not long enough to include it.

Until 1987, there had been much discussion about FinP, whether it was a protein or nucleic acid, how big it was, how strong its promoter was, etc., but no one had actually characterized the molecule. The first glimpse of FinP was provided by Walt Dempsey, who characterized the *traJ* and *finP* transcripts of R100 through Northern blotting, primer extension and RNase protection experiments (Dempsey, 1987). He determined that the FinP of R100 was a population of two molecules, one of approximately 105 bases, and one about 180 bases, initiating at a single site and terminating at different points, and F plasmid FinP was a single molecule of 105 bases. The *traJ* transcript in R100 was also a

Figure I.6 Diagram of F plasmid *finP* RNA. The RNA is presented as predicted by the RNAFOLD program in the PC/Gene (Release 6.5) package. Boxed nucleotides indicate bases that differ between *finP* alleles of F-like plasmids. The C* is the nucleotide altered in the *fisO* mutation.


Stem-Loop 1
Stem-Loop 2

collection of molecules, the longest being about 1050 bases. The shorter molecules were assumed to be produced by either premature termination or degradation of the *traJ* transcript. The results presented showed that FinO had a profound effect on FinP; when FinO was present in the cell, the amount of measureable FinP present increased about 10-fold. When *finP* was transcribed from the *tet* promoter of pBR322, the presence of *finO* increased the amount of *finP* transcript present in the cell and also caused an increase in the fidelity of the size of the chimeric transcript. Further, it was demonstrated that the presence of FinOP in R100-bearing cells caused a reduction in the amount of full-length *traJ* transcripts, and an increase in the amounts of truncated transcripts, ranging in length from 470-105 bases.

In a second paper published two years later, Dempsey proposed that *traM* transcripts, transcribed through their terminator into *traJ*, could function to derepress transfer by engaging FinP in 'nonproductive' RNA hybrids, titrating out FinP, leaving the *traJ* message free to be translated, and TraJ to promote transfer (Dempsey, 1989).

In a paper published in 1989, Frost et al. used modern molecular approaches to examine some of the classic problems first introduced by Willetts in the early '70's (Finnegan, Willetts, 1971). The *finP* and *fisO* mutants first described in 1971 (Finnegan, Willetts, 1971) were cloned into pUC18 and sequenced. The effect of these cloned *finP* mutants on the mating ability of both mutant and wild-type F-plasmids was measured. Using Northern blotting the relative amounts of mutant FinP molecules were determined.

The sequencing data showed that the *finP*⁻ mutations are mutations that decrease the calculated free energy of FinP's stems, making the molecule less stable, and the Northern blots showed that there was much less mutant *finP* RNA in a cell than wild-type *finP* RNA. The *fisO* mutation, mentioned previously as being a dominant mutation, involved the alteration of a critcal G:C base pair in the middle of stem 1 to a G:U base pair. This mismatch also resulted in a decrease in the amount of detectable *fisP* (the term for *finP* RNA with the *fisO* mutation is *fisP* RNA) RNA in the cell, but in this case the identity of

the C was the important factor in this mutation. If base-pairing was re-established by altering the G in the base pair to an A or if the RNA stem was re-stabilized by introducing a G:C base-pair directly adjacent to the *fisO* mutation, the amount of detectable *fisP* RNAwas not increased and the *fisO* phenotype was not relieved. The *fisO* phenotype seemed to be the result of competition of FisP with wild-type FinP's for FinO. Multicopy *fisO* will completely derepress a wild-type F plasmid in the presence of *finO*, and a simple *finP* mutant will not.

Walt Dempsey's observations that the amount of FinP in a cell was increased and that transcripts from exogenous promoters were more efficiently processed in the presence of *finO* were confirmed.

Recently, a third allele of *finP* was examined (Koraimann *et al.*, 1991). Koraimann et al. researched the FinP molecule of the F-like plasmid R-1. They showed that FinP was a small antisense RNA of 72 bases, and that the amount of FinP in R-1-containing cells increased in the presence of *finO*. They too performed site-specific mutagenisis on the *finP* gene and demonstrated that *finP* activity was not due to any polypeptide encoded by *finP*, for stop codons inserted in putative reading frames did not reduce its function. But mutations that altered bases in the loops, especially the second loop of FinP, did abolish its function. Using cloned *finP* genes they also demonstrated that *finP*, when overproduced by transcription from a synthetic lambda P_L promoter, could reduce plasmid transfer 100X in the absence of *finO*.

E. Experiments Described in This Thesis

This thesis addresses a number of aspects concerning the control of transfer in the F plasmid. First, the nature of the *traJ*RNA:FinOP interaction was investigated through the cloning and induced expression of *traJ* in the presence and absence of FinOP. Then, the repressive ability of either *finP* itself or its promoter was examined, by introducing a site-specific mutation into the F plasmid that abolishes *finP* transcription. The contribution of transcripts originating at promoters upstream of *traJ* to the control of repression was

evaluated by constructing a number of different transfer region clones and assessing their affect on F-bearing cells' transfer efficiency. Finally, the nature of FinOP interaction was studied by inducing *finP* expression in the presence and absence of *finO*, and noting any differences. The overall goal of this thesis was to begin a characterization of some of the molecular interactions involved in the repression of *traJ* expression by FinOP.

Chapter II. Methods and Materials

A. Strains and Media and Enzymes

Unless mentioned otherwise, all enzymes were supplied by Boerhinger Mannheim. Bacteria were grown on Luria-Bertani (LB) media (Sambrook *et al.*, 1989), sometimes supplemented with antibiotics to select for the plasmid or host strain of interest. M9 minimal salts medium was as described in Sambrook *et al.*(Sambrook *et al.*, 1989). Ampicillin was used at a concentration of 100 µg/ml, Kanamycin at 25 µg/ml, Streptomycin at 200 µg/ml, and Spectinomycin at 100 µg/ml. All antibiotics supplied by Sigma.

E. coli K12 strain JC3272 (F^- *lac* •X74 *his lys trp* Str^R $T6^R$) (Finnegan, Willetts, 1971) is the host strain for all F plasmids in this study, unless otherwise mentioned. M176 is JC3272 harboring the F'*lac* plasmid JCFLO; SL20 is JC3272 with SLF20, the site-specific *finP* mutant of JCFLO constructed in the experiments described below, and ED1864 is JC3272 containing the *fisO* mutant of JCFLO, EDFL68 (Finnegan, Willetts, 1971). *E. coli* ED24 is F^- *lac*$^-$ Sm^S Spc^R $T6^R$ $P1^R$, and is used as a recipient strain in mating assays. *E. coli* CQ24 (Edlund, et, 1986) is *ara leu lacI*^{q}::Tn5 *purE gal his argG rspL xyl ilv thi.*. *E. coli* WP146 contains EDFL50, which is JCFLO with the *traJ90* amber mutation, in the background *E. coli* strain ED 2601.

Table II.1 A table of the different strains of *Escherichia coli* employed in the experiments in this thesis

TABLE II.1
Escherichia coli Strains Used in Experiments

E. coli Strain	Genotype
JC3272	K12 F⁻ *lac* ϕX74 *his lys trp* Str^R $T6^R$
CQ24	F⁻ *ara leu* $lacI^q$::Tn5 *pur*E *gal his arg*G *rsp*L *xyl ilv thi*
ED24	F- Sm^S Spc^R $T6^R$ $P1^R$
ED2601	JC3272 *fla*

Table II.2 A table of the different F plasmids studied, and the strain designations of the bacteria that harbored them.

TABLE II.2
Escherichia coli Strains Harbouring F Plasmids

Strain	Host	F Plasmid	Distinguishing Feature
M176	JC3272	JCFLO	wild-type F plasmid; *lac*$^+$
SL20	JC3272	SLF20	P_{finP} mutant of JCFLO
ED1864	JC3272	EDFL68	*fisO* mutant of JCFLO
WP146	ED2601	EDFL50	*traJ* amber mutant of JCFLO

B. Recombinant Plasmids and Plasmid Vectors

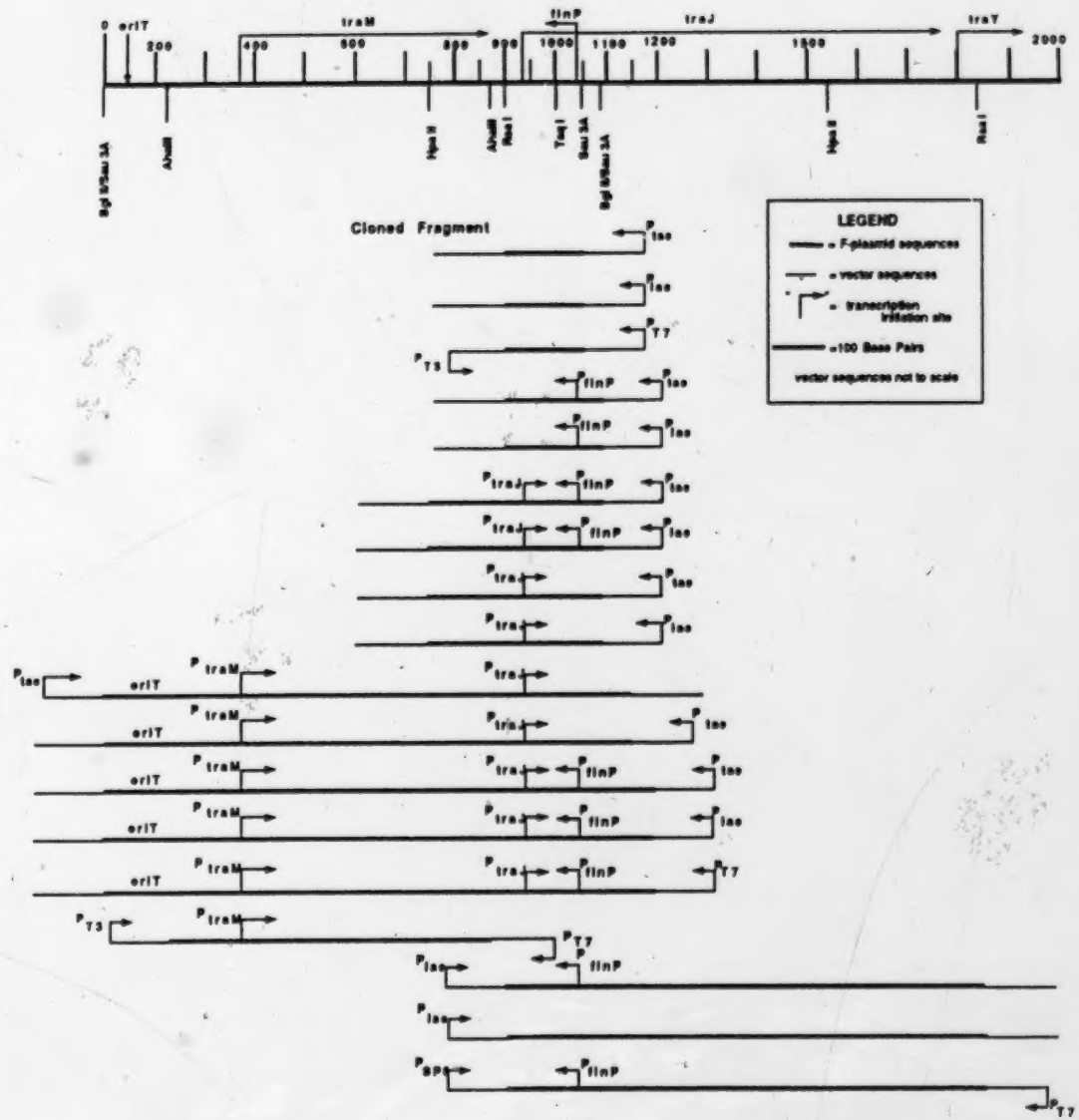
All recombinant plasmids used in this study are subclones from the 1.9 kb of DNA that includes *oriT* and ends with the end of *traJ* of the F plasmid. The boundaries and vector of each clone used in this study are as indicated in Figure II.1.

B.1 The Creation of pSQ1200, pSQ350, pSQ351 and pSQ180

pSQ1200 was created by transferring the *Bgl*II fragment which had been cloned into pNY300 into pTTQ18. This was done by digesting pNY300 (Frost *et al.*, 1989) with *Eco*R1 and *Hin*dIII in Boehringer Mannheim restriction buffer B (BoehringerMannheim Corp., 1989), incubating at 37° C for two hours, extracting the reaction with an equal volume of phenol, withdrawing the aqueous layer, and precipitating the DNA with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. The DNA was redissolved in autoclaved water, and combined with *Eco*RI-*Hin*dIII-cleaved pTTQ18 in a ligation reaction. The buffer used was Boehringer Mannheim ligase buffer (BoehringerMannheim Corp., 1989). About 25 ng of pTTQ18, and about 100 ng of pNY300 were added to the reaction. The ligation reaction was allowed to progress for 4 hours at room temperature, and then the ligation was used to transform *E. coli* JM109 cells, made competent by the $CaCl_2$ procedure (Sambrook *et al.*, 1989). Transformants were plated on LB agar containing 100 µg/ml ampicillin, and the resultant colonies were transferred to nitrocellulose filters and lysed as described in Sambrook *et al.* (Sambrook *et al.*, 1989). The filters were probed for F plasmid sequences with prPa labelled at its 5' terminus with ^{32}P, as described below. Colonies that harbored potentially positive clones were isolated, grown in liquid broth, and their plasmid DNA extracted by the small-scale method of Birnboim (Sambrook *et al.*, 1989). Positive clones were identified by digesting the candidates with *Eco*RI and *Hin*dIII, and electrophoresing them on agarose gels; standards of *Eco*RI-*Hin*dIII-digested pNY300 were run alongside the pSQ1200 candidates. Clones were considered positive if their *Eco*RI-*Hin*dIII fragments migrated the same distance as

Figure II.1 Map of all the cloned fragments of F plasmid DNA used in this thesis. The cloned fragments are aligned with their appropriate location on the map. Sequence information used to make this map from Dr. L. Frost, Dept. Microbiology, Univ. of Alberta.

Plasmid Name	Clone Boundaries	Vector
pSQ150	Rsa1-Sau3A	pTTQ18
pSnP99	Rsa1-Sau3A	pUC18
pSBP150	Rsa1-Sau3A	pBS
pSQ180	Rsa1-BglII	pTTQ18
pLF402	Rsa1-BglII	pUC18
pSQ350	HpaII-BglII	pTTQ18
pLF400	HpaII-BglII	pUC18
pSQ351	HpaII-BglII	pTTQ18
pLF401	HpaII-BglII	pUC18
pSQ1159	BglII-Sau3A	pTTQ19
pSQ1150	BglII-Sau3A	pTTQ18
pSQ1200	BglII-BglII	pTTQ18
pNY300	BglII-BglII	pUC18
pT7.300	BglII-BglII	pT7-3
pSBM650	AvaII-AvaII	pBS
pSJ89	RsaI-Rsa1	pUC18
pSJ88	RsaI-Rsa1	pUC18
pSJ39	RsaI-Rsa1	pGEM3Z



that of the fragment released by *Eco*RI-*Hin*dIII digestion of pNY300. The identity of positive clones was confirmed by sequencing them using the double-stranded DNA, dideoxynucleotide method described elsewhere (Frost *et al.*, 1989).

The other 'pSQ' clones mentioned in this section were constructed in the same way, through transferring *Eco*R1-*Hin*dIII-digested fragments from pUC chimeras, designated by the 'pLF' prefix, to pTTQ18. pSQ180 contains the *Rsa*I-*Bgl*II F plasmid fragment of pLF402, pSQ350 contains the *Hpa*II-*Bgl*II F plasmid fragment of pLF400, and pSQ351 contains the same *Hpa*II-*Bgl*II F plasmid fragment as pSQ350, but there is a site-specific mutation in the *finP* promoter; this fragment came from pLF401. All 'pLF' constucts were created by L. Frost. 'pNY' constructs were created by N. Yanchar.

B.2 Construction of pSQ1159

pSQ1159 was also created from pNY300. pNY300was digested with *Sau*3A, run on a 5% PAG, and was isolated from the gel with Maxam and Gilbert gel elution buffer (Sambrook *et al.*, 1989) as described below (III.B.5). The fragment was added in a ligation reaction to pTTQ18 which had been cleaved with *Bam*HI, extracted with phenol and precipitated with ethanol as described above. The two DNA fragments were present in the ligation reaction in a molar ratio of about 3 *Sau*3A fragments:1 *Bam*HI fragment. The ligation was transformed into *E. coli* JM109 and screened for F plasmid sequences as described above. All plasmids isolated contained the *Sau*3A fragment in an orientation opposite to that of the rest of the clones, with *oriT* aligned immediately downstream of the *tac* promoter. This clone was called pSQ1150. To create pSQ1159, a clone containing the F DNA fragment in the same orientation relative to the *tac* promoter as the rest of the clones, pSQ1150 was cleaved with *Eco*RI, *Pst*I, and *Ava*II, and ligated into pTTQ19 which had been cleaved with *Eco*RI and *Pst*I as described above. Positive clones were isolated and screened as described above, and the identity of pSQ1159 was confirmed by sequencing the 5' and 3' termini of the fragment as described elswhere (Frost *et al.*, 1989).

B.3 Creation of pSnP99 and pSQ150

pSnP99 was created by digesting pLF402 with *Sau*3A, isolating the 150 base pair fragment from a 5% PAG as described below (III.B.5), and ligating this fragment into pUC that had been cleaved with *Bam*HI. Positive clones were identified through the blue/white detection scheme of pUC (Yanisch-Perron *et al.*, 1985), plasmids were isolated as described above, and positive candidates were sequenced as described previously (Frost *et al.*, 1989). pSQ150 was created by transferring the F DNA fragment cloned into pSnP99 from pUC18 to pTTQ18 through *Eco*RI and *Hin*dIII digestion of pSnP99 and ligation of the 150 base pair fragment into pTTQ18 which had been cleaved by *Eco*RI and *Hin*dIII.

B.4 Plasmid Vectors

pUC18 is described by Messing (Yanisch-Perron *et al.*, 1985), pT7.3 and pT7.4 by Tabor (Tabor, Richardson, 1985), pTTQ18 and 19 by Stark (Stark, 1987), pGEM3Z by Promega (Promega Corp., 1989), and pBS by Stratagene (Stratagene Corp., 1990).

All strains grown carrying recombinant plasmids were grown in the presence of antibiotics at the concentrations indicated.

B.5 Cloning *traJ*

pSJ99 was constructed in the following manner: pLF119, containing the f6 *Eco*R1 fragment of the F plasmid, which spans the region from upstream of *oriT* to *traP*, cloned into pUC18, was digested with *Rsa*I and the restriction fragments were electrophoresed on a 5% PAG, along with pBR322/*Alu*I size standards. The third largest fragment, of apparent MW approximately 1kilobase (kb) was cut out of the gel, and the fragment was recovered by crushing the gel slice in 500 µl of Maxam and Gilbert elution buffer (Sambrook *et al.*, 1989) and soaking the slice overnight at 37° C, with agitation. It was then extracted with phenol until the interface was clean, and precipitated with ethanol at -20° C. It was then ligated into the *Sma*I site of pUC18, 19 (Yanisch-Perron *et al.*, 1985) and pGEM3Z (Promega Corp., 1989). The products of the ligation reaction were used to transform DH5α, which was plated on LB agar supplemented with ampicillin and Xgal

(0.02%). All white colonies tested contained clones bearing the *traJ* gene in an orientation 'facing away' from the *lac* promoter of both pUC18 and 19.

To construct a clone whose orientation was correct, an *Eco*R1-*Hin*dIII digest was performed on the *traJ* fragment cloned in pUC19. It was then ligated into *Eco*R1-*Hin*dIII digested pUC18, and used to transform JM109. Selection was on LB plates supplemented with only ampicillin. Oligonucleotide screening of transformant colonies transferred to nitrocellulose and lysed (Sambrook *et al.*, 1989) was used to identify positive clones. The orientation of the insert was checked through digestion with *Eco*R1 and *Bgl*II, and the fidelity of the ends of the insert determined through dideoxy sequencing of the double stranded DNA (Frost *et al.*, 1989).

C. RNA Isolation and Northern Blots

RNA was isolated and electrophoresed on agarose and polyacrylamide gels as previously described (Frost *et al.*, 1989). RNA was quantitated by fluorimetry using fluorimetry buffer (ethidium bromide at a concentration of 0.5 µg/ml,5 mM Tris-HCl pH 8, 1 mM EDTA, pH 8) and a Hitachi fluorimeter, model # F-2000 set at excitation 525 nm, emission 595 nm. The amount of fluorescence in a sample was converted to µg of RNA through the use of a Microsoft Excel program. The program contained the equation describing a standard curve of the amount of fluorescence given off by rRNA or tRNA samples versus the amount of RNA present in the sample, as determined by absorbance at 260 nm. When the fluorescence value of the unknown sample was entered, the program calculated the amount of RNA in the sample, using the standard curve's equation. RNA was transferred from gels to Zeta Probe (BRL) nylon membrane using a Hoeffer TE 50 electrotransfer lid and a TE series Transphor Electrophoresis Unit (model # TE42). The gel was prepared for transfer as described in the membrane supplier's instructions. Transfer was for at least 90 minutes - 30 at 15V and 60 at 25V - in 0.5X TBE, at 4° C. Probing

with polynucleotide kinase-labelled oligonucleotides was performed as described (Frost *et al.*, 1989).

D. *In vitro* transcription and probing of blots with radiolabelled RNA probes

D.1 *In vitro* Transcription Reaction

The plasmid of interest (0.5-1 µg) was linearized with the appropriate enzyme (see D.3), then extracted with phenol and precipitated with three volumes of ethanol. After at least 30 minutes at -20° C the precipitated plasmid was pelleted for 15 minutes in a Beckman 'Microfuge E' microfuge at 4° C. The pellet was rinsed with 70% ethanol, then dried in a Savant Speed Vac concentrator (model #SVC100H) for 3-5 minutes. The DNA was then dissolved in the reaction mixture. The transcription mix was as per Promega Biotech (Promega Corp., 1989), with some minor modifications: the 5X *in vitro* transcription buffer was according to Boerhinger Mannheim (BoehringerMannheim Corp., 1989), and the radiolabel was the only source of CTP. α^{32}P-CTP was from New England Nuclear and was delivered with a specific activity of 800 Ci/mmol, and a concentration of 40 µCi/µl; 4 µl were added to each reaction. Transcription was allowed to progress for 45-60 minutes at 37° C, then 27 units of RNase-free DNase were added and allowed to digest the template for 15-20 minutes. T7, T3 RNA polymerases and DNase were from Boerhringer Mannheim. RNAguard was from Pharmacia.

The probe was purified through a Nuctrap column (Stratagene), and 1 µl was quantitated by liquid scintillation counting using toluene based counting fluid supplemented with POPOP and PPO. Radioactive quantitation was in a RackBeta counter from LKB. Finally, the integrity of the probe was tested by denaturing PAGE of a 1 µl sample followed by autoradiography .

D.2 Hybridization and Washing of Blots

Blots were prehybridized for at least 3 hours at 58° C in 50% formamide, 2.5X SSC (Sambrook *et al.*, 1989), 5X Denhardt's solution (Sambrook *et al.*, 1989), 1.5% SDS, and 100 µg/ml of Sigma *E. coli* Strain W tRNA type XX . The blots were probed at 58° C with 10^6 cpm/ml hybridization solution in a fresh batch of the same buffer except with the addition of 200 µg/ml calf thymus DNA (Sigma) that had been boiled at least 10 minutes, and 200 µg/ml tRNA. The blots were hybridized for at least 9 hours before washing. Washing was as follows: a 5 minute rinse in 2X SSC at room temperature, a 10 minute wash in 2X SSC, 0.1% SDS, a 10 minute wash in 0.5X SSC, 0.1% SDS at room temperature, and a final wash of 10 minutes in 0.1X SSC, 0.1% SDS, at 55° C. Exposure was at -70° C in the presence of a Fisher Biotech 'lightning plus' intensifying screen, using Kodak X-AR5 film.

D.3 DNA Templates Transcribed to Generate RNA Probes

The initial probe for *traJ* mRNA was a T7 transcript of pT7.300 linearized with *Hin*dIII; *in vivo* T7 transcription suggested that this transcript would contain only the *finP* sequences, as the second stem-loop of *finP* seems to be a termination signal recognized by T7 RNA polymerase. The full length *traJ* mRNA probe was a T7 transcript made from pSJ39 linearized with *Hin*dIII. The second 5' specific probe was a T7 transcript of pSBP150 linearized with *Hin*dIII. It was complementary to the first 120 bases of the *traJ* mRNA. The probe specific to the 3' end of *traJ* mRNA was a T7 transcript of pSJ39, linearized with *Ssp* 1, which cuts *traJ* DNA at nucleotide 638 (relative to the start of the mRNA transcript), and is complementary to the 3' 200 bases of the *traJ* transcript.

E. Oligonucletotide Probes

The oligonucleotide used to detect *traJ* mRNA was prJ1 -5' TTAACGTGGCATT-AATTGGATA 3', complementary to nucleotides 7-22 in the *traJ* transcript.

The oligonucleotide used to detect *finP* RNA was prPa - 5' GAGGTTCT*TAT-GTATC 3', complementary to the first 12 bases of finP, with one mismatch at the indicated position.

The oligonucleotide used to mutagenize the *finP* promoter was TATGCTG* G-GTAGCCT, the G* changed the invariant T in the -10 region of the promoter to a C.

F. IPTG Inductions

Cells were grown to an OD_{600} of 0.5-0.75. IPTG (Sigma) was added to a final concentration of 1mM from a 100mM stock solution, and at appropriate intervals, 1 ml samples were withdrawn and added to ice-cold tubes containing 10 µl of 1 M sodium azide and 40 µl of 10 mg/ml chloramphenicol. The cells were kept on ice until the experiments' end, then centrifuged and the cell pellets quick frozen at -70°C. RNA isolation was by hot phenol method as previously described (Frost *et al.*, 1989).

In the case of rifampicin addition, rifampicin (Sigma) was added to a final concentration of 200 µg/ml, from a 100X stock of 20 mg/ml dissolved in HPLC grade methanol. The rifampicin was made fresh before each experiment.

G. In Vitro Mutagenesis

In vitro mutagenesis of double-stranded pUC chimeras was as described previously (Frost *et al.*, 1989).

H. Phage Sensitivity Tests

To determine bacteriophage sensitivity, two different methods were used. Bacteria from a number of colonies of the same strain were smeared over the face of an LB plate, seeding a lawn. Then a 2 µl drop of R17 phage ($5x10^{11}$ pfu/ml) was placed in the middle of the lawn. The bacteria were incubated at 37° C to detect plaques in the bacterial lawn. This method was used in the complementation tests of pSJ99. The second method used a

sterile pasteur pipette containing phage suspension to streak a line of phage across the face of an LB plate. Then, using loops dabbed on a single colony, lines of bacteria were streaked perpendicular to and intersecting the line of phage. If the bacteria were sensitive, there would be no growth of the bacterial streak after contact with the phage line. This technique was used in the screening of SL20 candidates.

I. SLF20 Creation

M176 was grown aerobically to mid-log phase and 100 µl of the culture was added to 800 µl of LB-broth. Then 100 µl of mid-log phase ED24 containing pED104 and pLF401 was added to the M176. The two strains were mixed gently and allowed to incubate at 37 ° C without agitation for 30 minutes. 10 µl of the mating mixture were diluted 1000-fold in 1X SSC (Sambrook *et al.*, 1989), and 10 lots of 100 µl were plated on 75 mm diameter petri plates (Fisher) of L1 medium, which is M9 minimal salts medium (Sambrook *et al.*, 1989) supplemented with 0.16% lactose and 50 mM $MgSO_4$. Kanamycin, ampicillin, and spectinomycin were added at concentrations specified above. The plated bacteria were incubated for two days at 37° C, then the colonies were scraped off the plates and used to inoculate 50 ml of LB broth. This culture was grown to mid-log phase, and used to mate into a JC3272/pED104 recipient strain, using the protocol described above. 100 µl of the mating mixture was used to inoculate 150 mm petri dishes(Fisher) containing L1 medium supplemented by kanamycin and streptomycin. The bacteria were incubated for 2 days at 37° C, and then the colonies were transferred to nitrocellulose and lysed (Sambrook *et al.*, 1989). These colony lifts were probed with the primer that coded for the mutation labelled with ^{32}P by T4 polynucleotide kinase at its 5' terminus. They were probed at room temperature, and then washed twice in 6X SSC, 0.5% SDS at room temperature, and used to expose X-ray film. After the filters had given a reasonable signal on the film, they were washed again, this time at a temperature 5° C lower than the calculated melting temperature of the oligonucleotide for 10 minutes at an

ambient temperature of 37° C, and finally for 2 - 5 minutes at the oligonucleotide's melting temperature, monitoring the wash with a Geiger counter, to ensure that all the radioactivity had not been washed off. The filters were then re-exposed X-ray film, and regions of the blot that maintained their signal were noted. The remains of the colonies on the petri dishes that corresponded to the areas of intense signal were re-streaked, and these bacteria were tested for phage sensitivity.

One colony tested phage sensitive in the presence of *finO*. To verify the nature of the mutation, F plasmid DNA was isolated from the potential mutant, using the method described by Skurray et al. (Skurray *et al.*, 1976). This DNA was digested with *Sau*3A and *Rsa*I, and run on a 5% PAG along with samples of JCFLO DNA, pLF400 and pLF401, all digested with *Sau*3A and *Rsa*I. These digests were electroblotted to Zeta-Probe membrane, and probed for the *finP* sequence, to determine whether the desired mutation had been incorporated.

J. Mating Assays

Mating assays were performed as described by Frost et al.(Frost *et al.*, 1989).

Chapter III. FinOP and *traJ* Expression

A. Introduction

F-like plasmids are a group of related large (>90kb) plasmids that inhabit strains of the bacterium *Escherichia coli*. (Finnegan, Willetts, 1971). Members of this family encode the ability to transfer themselves to plasmidless bacteria through a process mediated by cell-to-cell contact called conjugation. The functions necessary to carry out conjugation are numerous, and up to 35kb of F-like plasmids' genomes are devoted to encoding these functions (Willetts, Skurray, 1987).

In the majority of bacteria carrying these plasmids the conjugative ability of the resident plasmid is repressed by a fertility inhibition, or *fin* system, which consists of the gene products of *finO* and *finP* (Finnegan, Willetts, 1971). Both are required for full repression of transfer. FinP has been shown to be an antisense RNA molecule complementary to the 5' untranslated region of the mRNA of *traJ* (Dempsey, 1987), the positive regulator of the transfer operon (Finnegan, Willetts, 1973). FinO has been tentatively identified as a protein (Timmis *et al.*, 1978; McIntire, Dempsey, 1987; Yosioka *et al.*, 1987). FinOP have been shown to repress the expression of *traJ* at the transcriptional level (Willetts, 1977).

Although it has been shown that FinOP reduce the amount of *traJ* mRNA in F-like plasmid bearing cells (Willetts, 1977), the question of how the repression is performed has not been addressed experimentally. Antisense RNA's are known to use a number of different mechanisms to repress gene expression. RNA-OUT, produced by the transposon Tn10, hybridizes with its target mRNA's ribosomal binding sequence and prevents the translation of the messenger RNA(Ma, Simons, 1990). This interaction also reduces the half-life of the mRNA it recognizes (Case *et al.*, 1990b). OOP RNA, produced by phage lambda, binds to the 3' end of its target sequence, and through this binding introduces an RNaseIII cleavage site to the mRNA (Krinke, Wulff, 1987). The mRNA is endonucleolytically cleaved by RNase III and then quickly degraded. Finally, the *crp* (cyclic AMP receptor protein) gene is controlled by an antisense transcript that appears to bind to the DNA sequence of the target gene near the promoter area, making it untranscribable (Okamoto, Freundlich, 1987). FinP is complementary to the region of the *traJ* transcript that contains the ribosome binding site of *traJ*. Thus, Mullineaux and Willetts proposed that FinP reduces *traJ* expression by hybridizing with *traJ* mRNA and occluding ribosomes from the ribosome binding site (Mullineaux, Willetts, 1985). Dempsey went on to propose that FinP has a polarity-like effect, where the prevention of translation results in premature termination of transcription (Dempsey, 1989). The evidence for that idea was that cells containing *finOP* contained fragments of *traJ* mRNA much smaller than the full length transcript.

The experiments performed in this chapter attempt to address the question of the mechanism of FinOP repression of *traJ* expression. First, it was confirmed that the reduction of *traJ* mRNA reported in the literature was observable on Northern blots performed in our laboratory. *traJ* was cloned in pUC18, which placed its production under the control of the *lac* promoter. Then, a site-specific mutant of *traJ* was created. The mutation was derived from the *finP* promoter sequence of the *finP*$^-$ F-like plasmid R386 (Finlay *et al.*, 1986). The mutation destroyed the activity of the *finP* promoter without

altering the sequence of the *traJ* protein . Using pUC clones of this mutant as well as the wild-type *traJ*, transcripts were produced by induction of the *lac* promoter with IPTG. The results suggested that FinP or the transcription of *finP* had a drastic reductive effect on *traJ* expression. To confirm this observation, the mutation was recombined into the F plasmid, and this mutant plasmid was tested for conjugative activity and *traJ* mRNA levels. There was much more *traJ* mRNA produced in the mutant F plasmid, but not to the same extent as observed with the cloned *traJ*. This increase of *traJ* mRNA did not have any significant effect on the conjugative properties of the bacterial strains harboring the mutant F plasmid.

B. Results

B.1 Northern Blotting

Two types of probes were used to detect *traJ* mRNA on Northern blots - oligonucleotides and *in vitro* transcribed ^{32}P-labelled RNA transcripts. Only single-stranded probes could be used in these investigations, because RNA molecules encoded by both strands of the DNA were present in the samples, and the data generated by double-stranded probing would lead to confusion regarding the origin of the detected species.

An RNA probe complementary to the 3' 200 bases of the *traJ* transcript was used with success; a representative autoradiogram of a probed blot is shown in Figure III.1. In this Figure, a blot of a 25 cm, 1.5% agarose gel, a number of bands were recognized by the anti-*traJ* mRNA probe. These bands are visualized as distinct because the gel is longer and the bands are resolved from rRNA. The lowest band of apparent MW approximately 0.95kb corresponds to the *traJ* transcript. There are a number of bands in the M176 lane that are larger than the *traJ* transcript - these must represent read-through transcripts into the *traY* gene, because a probe complementary to the traM gene did not hybridize to the same RNA species.

This blot shows the effect of *finOP* upon the abundance of *traJ* message. There is an almost complete abolition of all *traJ* RNA species present in M176; a small amount of the approximately 2 kb transcript is detectable. The origin of the collection of smaller transcripts and the one of apparent length 2.5 kb detected in the M176/pED104 lane is probably from the vector carrying the cloned *finO*, pACYC177, which produces transcripts that hybridize to the *traJ* probe used (data not shown).

An oligonucleotide probe complementary to nucleotides 7-22 of the *traJ* transcript was constructed and it was used successfully in Northern blots of strains containing cloned fragments of *traJ*, but did not prove sensitive enough to detect *traJ* transcripts produced by the wild-type F plasmid (data not shown).

B.2 Cloning *traJ*

After the initial probing of Northern blots with the radiolabelled FinP molecule that yielded the results in agreement with published data, the question that was asked was: at what stage of the *traJ* mRNA's existence does the FinOP system work? To answer this question properly, it would be necessary to clone the *traJ* gene. Previously, Mullineaux and Willetts (1985) had found that multi-copy plasmids containing *traJ* were unstable, and were lost from cells or mutated or recombined. Therefore it was decided that the structural gene must be cloned without its promoter. The fragment containing only the structural gene for *traJ* would be cloned into the vector pUC18/19, which contains the controllable *lac* promoter upstream of its multiple cloning site. The resulting contruct then would contain the structural gene for *traJ* but its expression would be tightly regulated. The cloning strategy is diagrammed in Figure III.2.

The first screening for transformants was performed in *E.coli* DH5α, which allows for leaky expression of the *lac* promoter in the absence of IPTG. All white colonies picked contained clones with the *traJ* structural gene opposing the *lac* promoter, in both vectors pUC18 and pUC19. This result confirms previous reports of difficulty in cloning an expressed *traJ* gene. The *traJ* gene was then cloned into pUC18, taking advantage of the reverse orientation of the pUC18 and 19 multiple cloning sites with respect to P_{lac} (Yanisch-Perron *et al.*, 1985)to obtain a *traJ* gene cloned in the same orientation as the *lac* promoter. To repress all expression from the P_{lac} of pUC, the new constructs were transformed into JM109, which carries the *lacI*^{q} mutation, which overproduces the repressor of P_{lac}. Positive transformants were selected by colony blot hybridization, since blue/white selection could not be used, as the expression of *traJ* was not desired. All chimeras contained *traJ* in the proper orientation. This clone, pSJ99, has been stable and maintained without any instances of recombination or excision.

Two potential problems had to be considered before the cloned *traJ* could be used in the study of *traJ* mRNA/FinOP interaction. One possibility was that translation of the β-

Figure III.1 The effect of FinOP on the amount of *traJ* RNA in a cell. This figure is an autoradiogram of a Northern blot of total RNA from strains indicated, probed for *traJ* mRNA. The probe was an internally labelled, *in vitro* transcribed transcript complementary to the 3' 200 bases of *traJ* mRNA. Equivalent amounts of RNA were added to each lane, about 25 μg per lane. The position of RNA size standards is indicated by the black dots, their size is given in kilobases. The arrows point to *traJ* transcripts discussed in the text. Bands between the two species are taken to be degradation products of the largest band.

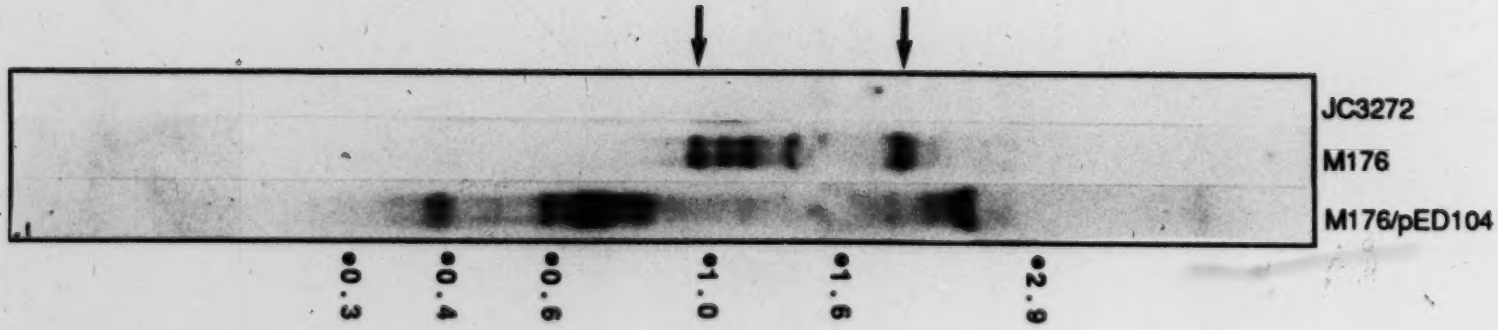
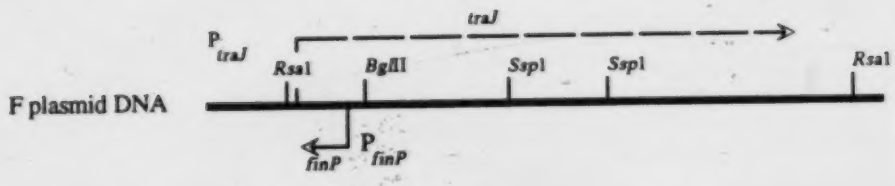
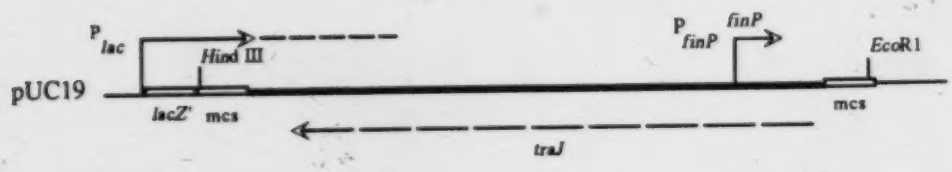

JC3272
M176
M176/pED104
0.3
0.4
0.6
1.0
1.6
2.9

Figure III.2 Cloning scheme used to construct pSJ99. Arrows represent genes; the vertical lines connecting with them indicate transcription starts.



digest with *Rsa*I
ligate into *Sma* site of pUC19



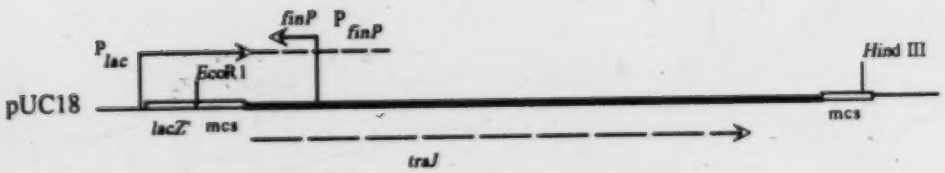
Digest with *Eco*R1 and *Hin*dIII

Clone into pUC18

transform into *lacI* ^{q}cells

in the absence of IPTG

select positive clones by probing colony blots for *traJ*



The *traJ* gene is now in the same direction as transcription originating from the inducible *lac* promoter

0.1kb

vector sequences
not to scale

galactosidase gene encoded by *lacZ'* in pUC may continue through the untranslated leader region of *traJ*, thus protecting the mRNA from interaction with FinP. The second possibility was that the additional *lacZ'* mRNA might have regions of homology with the *traJ* message that would hybridize with it and thereby interfere with the formation of RNA secondary structure recognizable by FinP. Either of these scenarios would prevent the cloned *traJ* message from being repressed by FinOP. Sequence analysis showed that *lacZ'* would encounter a stop codon within the first ten nucleotides of the *traJ* transcript, and the mRNA would be free to interact with FinP. RNA secondary structure predictions indicated no significant change in mRNA folding of the chimeric message. But to be sure, the following experiment was performed. The clone was transformed into an *E. coli* strain that contained an F plasmid which had a mutation that caused premature termination of translation of *traJ* (*E. coli* WP146). Two strains were constructed from this background strain; one contained pED104 in addition to the cloned *traJ*, while the other contained only cloned *traJ*. These strains were grown on media that contained 0.2% lactose, which is a weak inducer of the lac operon, and tested for sensitivity to F-pilus specific phage. The host strain was insensitive to the F pilus-specific phage because the mutation in the F plasmid abolished production of TraJ. Therefore there would be no TraJ available to enhance the expression of the transfer operon, and no F pili would be produced. The cloned *traJ* re-established phage sensitivity to the strain containing the mutant F plasmid; it complemented the mutation in *traJ*. When both *finO* and cloned *traJ* were present in the strain, the bacteria were insensitive to the phage. The cloned gene's expression was repressed by the wild-type fertility inhibition system. Since the cloned gene could complement a null mutation and was repressed by FinOP it was concluded that it would serve as an adequate model for the study of FinOP/*traJ* mRNA interaction.

B.3 Induction of *traJ* Expression

To obtain all possible combinations of repression gene products, a *traJ* gene with no *finP* was required. A mutation that eliminated all transcription of *finP* was made in the

finP promoter through site-specific mutagenesis, based on the *finP* mutation of the plasmid R386 (Finlay *et al.*, 1986). This mutation was demonstrated to abolish transcription from P_{finP}; extended exposures of Northern blots probed for FinP did not yield any FinP band (Fig. III.3). This construct was named pSJ88. The strong hybridization to 16S rRNA observed is due to non-specific hybridization of prPa with the rRNA. This background is seen because the blot was not washed at a very stringent temperature, to detect *finP* RNA with as much sensitivity as possible.

The FinP in the system was contributed from the *finP* gene present on pSJ99. *finO* was supplied from pSnO104, a construct analogous to pED104, but employing the vector pACYC184 to host the *finO* gene rather than pACYC177. The strain *E. coli* CQ24, which contains the $lacI^q$ gene on an integrated Tn5 construct, was used to ensure that sufficient LacI is produced to repress the *lac* promoter.

To study the process of FinOP repression of *traJ* expression, the following experiment was devised (Fig. III.4). Cells would be grown to early log phase, then the production of *traJ* mRNA would be induced through the addition of IPTG, which induces the transcription of the *lac* promoter of pUC. Timed samples would be removed, and the state of the *traJ* mRNA would be monitored through Northern blots.

The four combinations of repressor genes (neither, *finO* alone, *finP* alone, *finOP*) were tested for their effect on the production of *traJ* transcripts from the P_{lac} of pSJ99 and pSJ88. Both cells with *traJ* alone, or with *traJ* and *finO* induced extremely efficiently, as shown in Figure III.5 in the lanes containing the RNA produced by pSJ88 and pSJ88/pSnO104. When *finP* alone was present (pSJ99), the amount of induced *traJ* mRNA was reduced by at least 100-fold from that containing no *finP* (pSJ88). The addition of *finO* to the system further reduced the total amount of *traJ* mRNA transcribed, and was more efficient than *finP* at blocking the production of full-length 920 base *traJ* transcripts (pSJ99+pSnO104). No significant differences in the pattern of bands that represent incomplete *traJ* transcripts was observed. If FinOP were acting at a post-

Figure III.3 Absence of detectable FinP transcribed from a P_{finP} mutant. This figure is an autoradiogram of a Northern blot of total RNA probed for *finP* RNA. The probe was an oligonuclueotide complementary to the first 13 bases of FinP, labelled at its 5' end with ^{32}P. Lane 1: 10 µg of RNA extacted from CQ24 cells containing pSJ99. Lane 2: 10 µg of RNA extracted from CQ24 cells containing pSJ88.

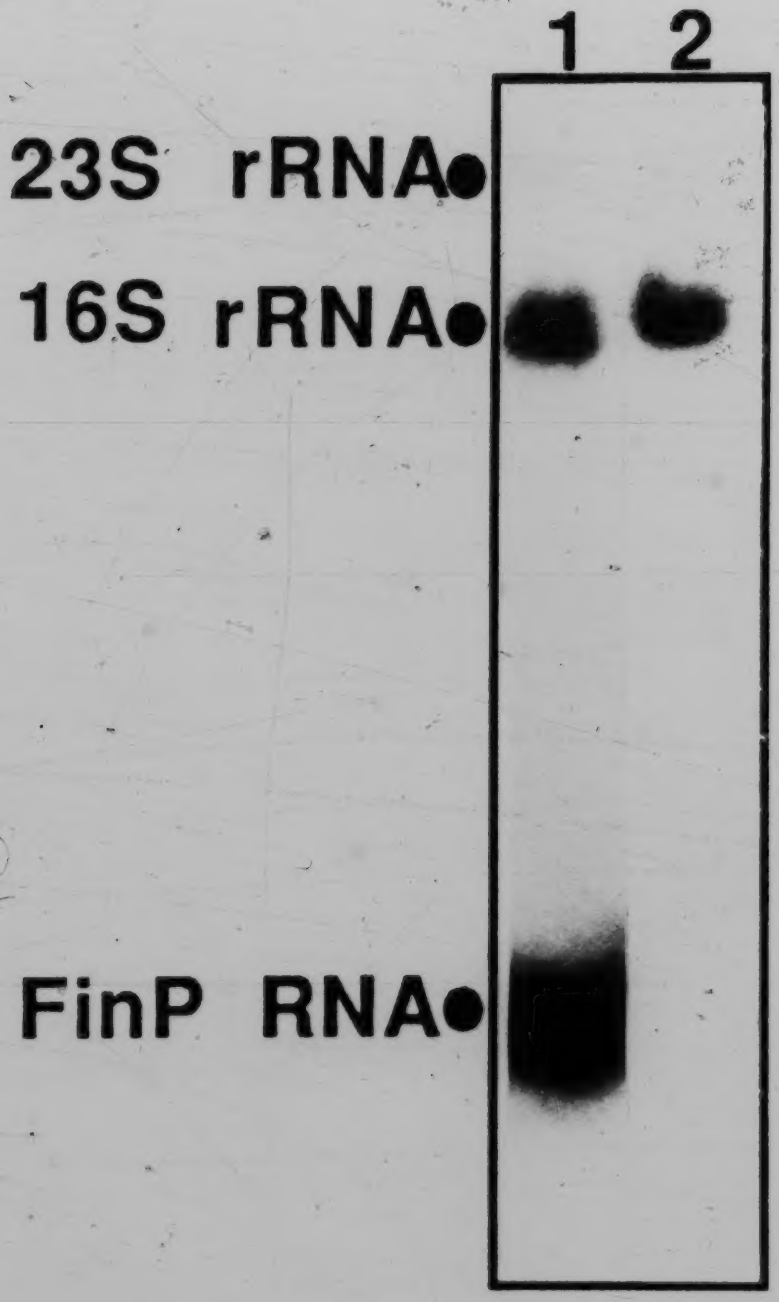
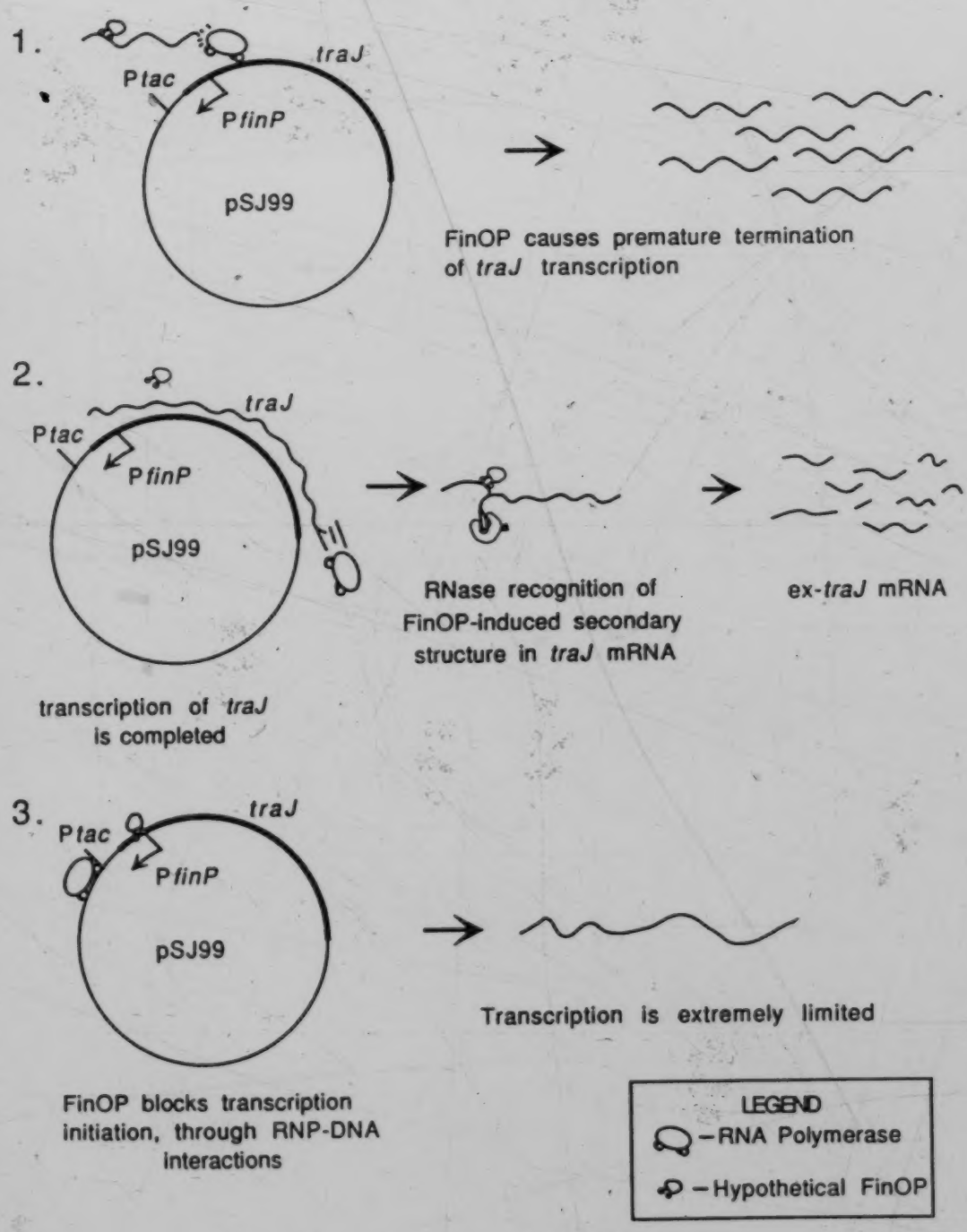

1 2
23S rRNA
16S rRNA
FinP RNA

Figure III.4 A diagram outlining the expected consequences of three different antisense RNA paradigms in the experiment described in the text.
A. Hypothetical scenarios.
B. Schematic representation of predicted autoradiograms for each hypothetical mechanism.

A. INDUCTION EXPERIMENTS-PROBABLE SCENARIOS



B. Diagram of Expected Autoradiograms of Northern Blots probed for traJ RNA in Each Different Repression Scenario

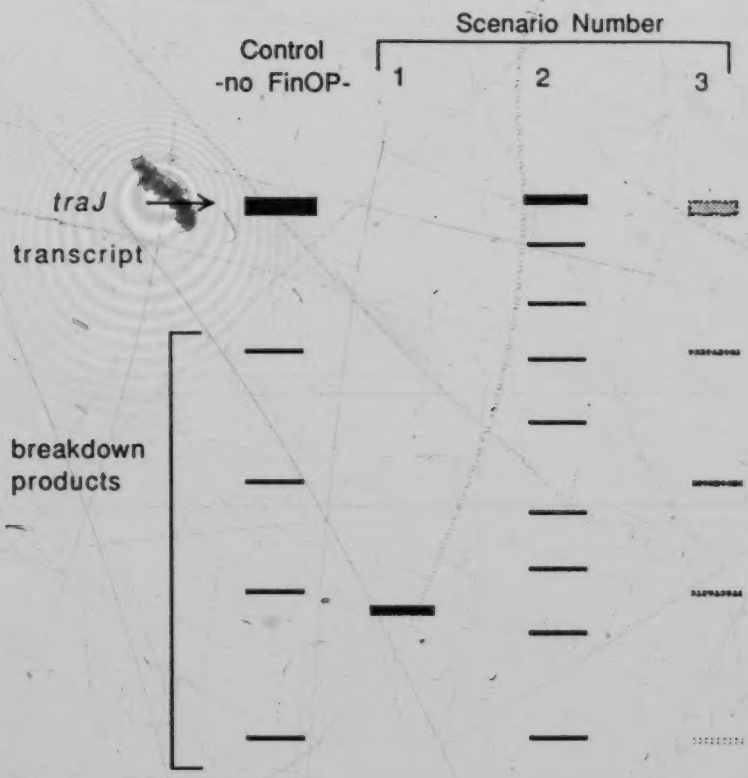


Figure III.5 The effect of *finP* on transcription of *traJ* induced from a *lac* promoter under *lacI*^{q} control, with and without *finO*. This figure is an autoradiogram of a Northern blot of total RNA from the strain CQ24 containing the plasmids indicated in the figure. pSJ99 is the wild-type *traJ* gene cloned into pUC, and pSJ88 is a site-specific mutant that produces no FinP (see text). The blot has been probed for *traJ* with an *in vitro* transcribed internally ^{32}P-labelled RNA probe comlementary to the entire *traJ* mRNA. Dots indicate the position of RNA size standards, their size is given in bases. Arrows indicate positions of major truncated *traJ* RNA species, the numbers next to them indicate the species' estimated size, in bases. '-I' indicates the RNA sample was collected before IPTG induction; The numbers above each lane indicate the time after induction that the sample was withdrawn at, in minutes. The two samples on the left represent autoradiograms obtained with a shorter exposure of the same blot that yielded the portion of the figure on the right, while the two pSJ88 and pSJ99 samples in the right-hand portion of the figure represent an autroradiogram that is overexposed for the pSJ88 samples in order to visualize the bands on the pSJ99 samples. The band whose size is estimated as 920 bases represents the putative normal *traJ* transcript; all larger bands represent read-through transcription into vector sequences.

FinP Reduces the Expression of traJ RNA from a Fully Induced lac Promoter

transcriptional level, then it would be expected that there would be a difference between transcripts terminating and degrading through cellular mechanisms and those doing so with the aid of FinOP. This difference would be seen on the autoradiogram as a difference in the pattern of bands of lower molecular weight *traJ* mRNA (see Fig. III.4 B). Also, one would expect to see the 80 base *lacZ* transcript + the 100 base *traJ* 5' region complementary to *finP*, as the induced *lac* promoter facilitated transcription very efficiently, and much *traJ* mRNA was produced. There were no major bands in that size range observed. This observation led to the conclusion that some component of *finP* - either the transcript or the act of its transcription - was blocking the transcriptional initiation of the *lac* promoter, 180 bases upstream. There is the possibility, though, that any incomplete transcripts may be too unstable to be detected by this type of procedure.

When blots containing RNA generated from induction of pSJ99 were probed for FinP, there was no noticeable change in FinP band intensities over time (data not shown).

Experiments were performed where rifampicin was added after a burst of *traJ* RNA induction from pSJ88 or pSJ99, timed samples were removed after the rifiampicin addition, RNA was extracted and run on denaturing PAGs, electroblotted to Zeta Probe and probed for *traJ* RNA. The experiments showed that *finOP* does not significantly catalyze the degradation of, and does not change the pattern of the *traJ* RNA degradation intermediates under the experimental conditions described above (data not shown).

B.4 Creation of SLF20, and its Comparison to JCFLO

The induction experiments demonstrated that *finP* had a profound effect on the transcription of *traJ*, but because the experiment was performed in a multicopy plasmid and not the F plasmid, there was a chance that this observation could be an artifact of the experimental design. To examine this possibility, a similar *finP*$^-$ mutant would have to be created in the F plasmid, to demonstrate whether or not this mutation has a similar effect on the production of *traJ* transcripts. This mutation was created by mating the F plasmid into cells containing the *finP*$^-$ promoter mutation on a fragment of F DNA cloned into pUC18.

pSJ88 could not be used, because it would supply background levels of TraJ that would sabotage the selection system employed, instead a plasmid (pLF401) carrying only the 5' region of *traJ* was chosen. Also present in the cells was *finO*, on a multicopy plasmid. The following strategy was used to create the *finP*⁻ F plasmid mutant (Fig. III.6).

The wild-type F plasmid was mated into a strain carrying multicopy plasmids encoding both the mutation of interest and *finO*. During the conjugation of the wild-type F into this strain, it was hoped that the incoming F plasmid would recombine with the mutated F DNA on pLF401 and incorporate the mutation. Then a second mating would be performed to transfer the newly mutated F-plasmid out of a cell that had many copies of the same mutation present on a pUC chimera, to one with no copies of pLF401, so that the F plasmid would be detectable. The second recipient strain also contained *finO*, on pED104. The transconjugants from this second mating were grown on media that selected for recipients containing the F plasmid, then the colonies were transferred to nitrocellulose, and their DNA probed with the oligonucleotide that was used to construct the original mutant. Colonies whose signals persisted through stringent washes were then tested for sensitivity to F-pilus-specific bacteriophage. Since *finO* was present, and would combine with the plasmid's FinP and repress pilus formation, F plasmid-bearing cells should be phage resistant. Thus, a phage sensitive colony would be deficient in the FinOP system, and was probably carrying the *finP* mutation recombined into the plasmid. This test yielded one positive mutant. Its identity was confirmed as the mutation of choice by restriction digestion with *Rsa*I and *Sau*3A, Southern blotting, and *finP*-specific oligonucleotide probing of F plasmid DNA (Fig. III.7). A *Sau*3A site was destroyed by the promoter mutation, and therefore *finP* was present on a fragment of DNA of 180 bases in the mutant and 150 bases in the wild-type.

This mutant F plasmid was called SLF20, and the *E. coli* strain carrying it was named SL20 (JC3272/SLF20). The purpose of creating SLF20 was to determine if the effect of the *finP* promoter or FinP alone observed in the IPTG induction of pSJ99 was a true one or

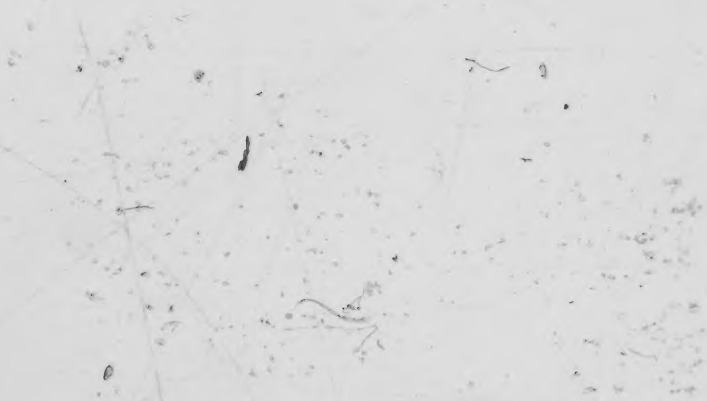


Figure III.6 A diagram of the strategy used to create SLF20. The large ovals represent individual bacteria, the solitary rectangular structures represent pili, and different background fill patterns indicate different bacterial strains. The circular figures with little teeth represent F-pilus-specific bacteriophage.

The Construction of SLF20 - a *finP* Promoter Mutant

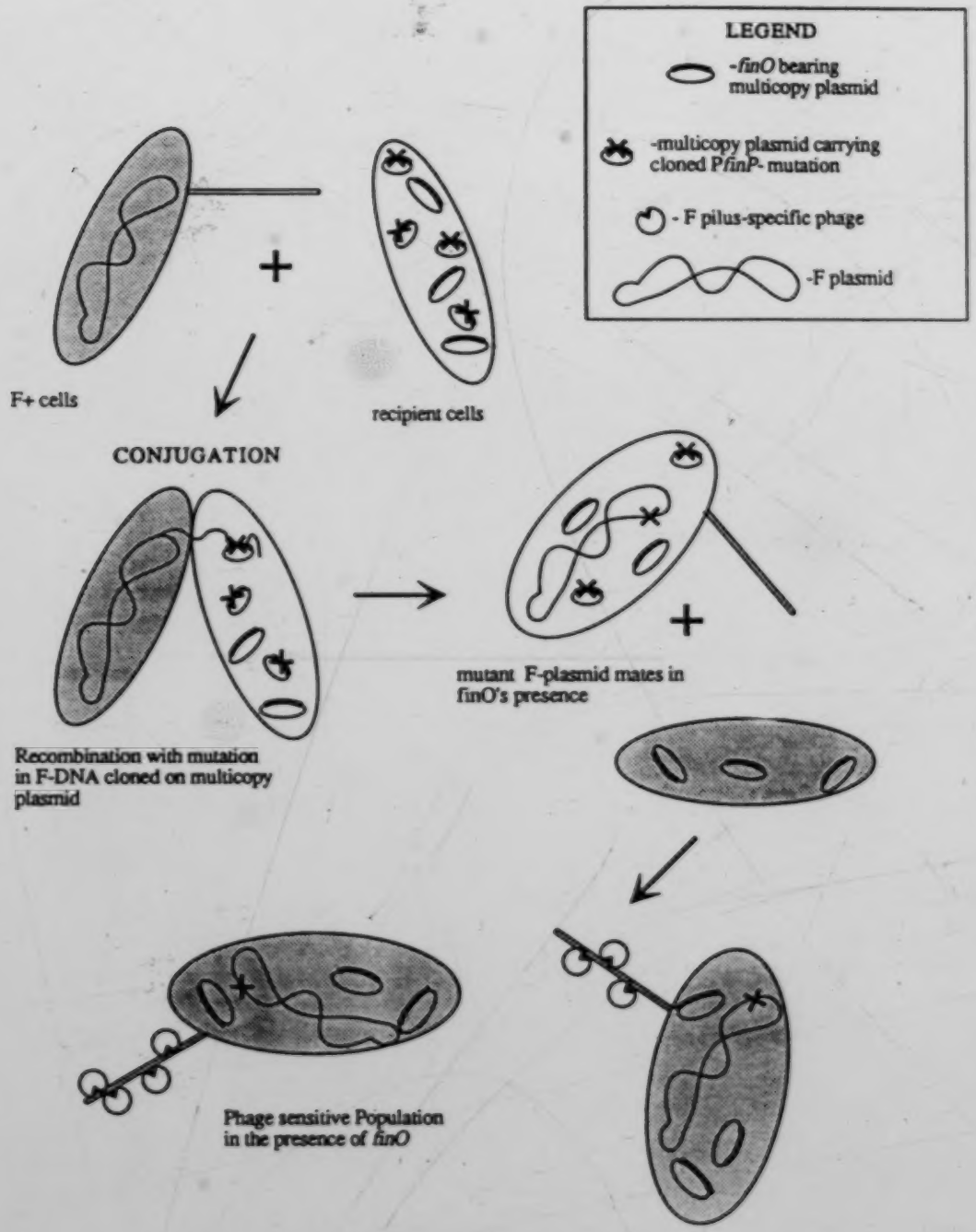
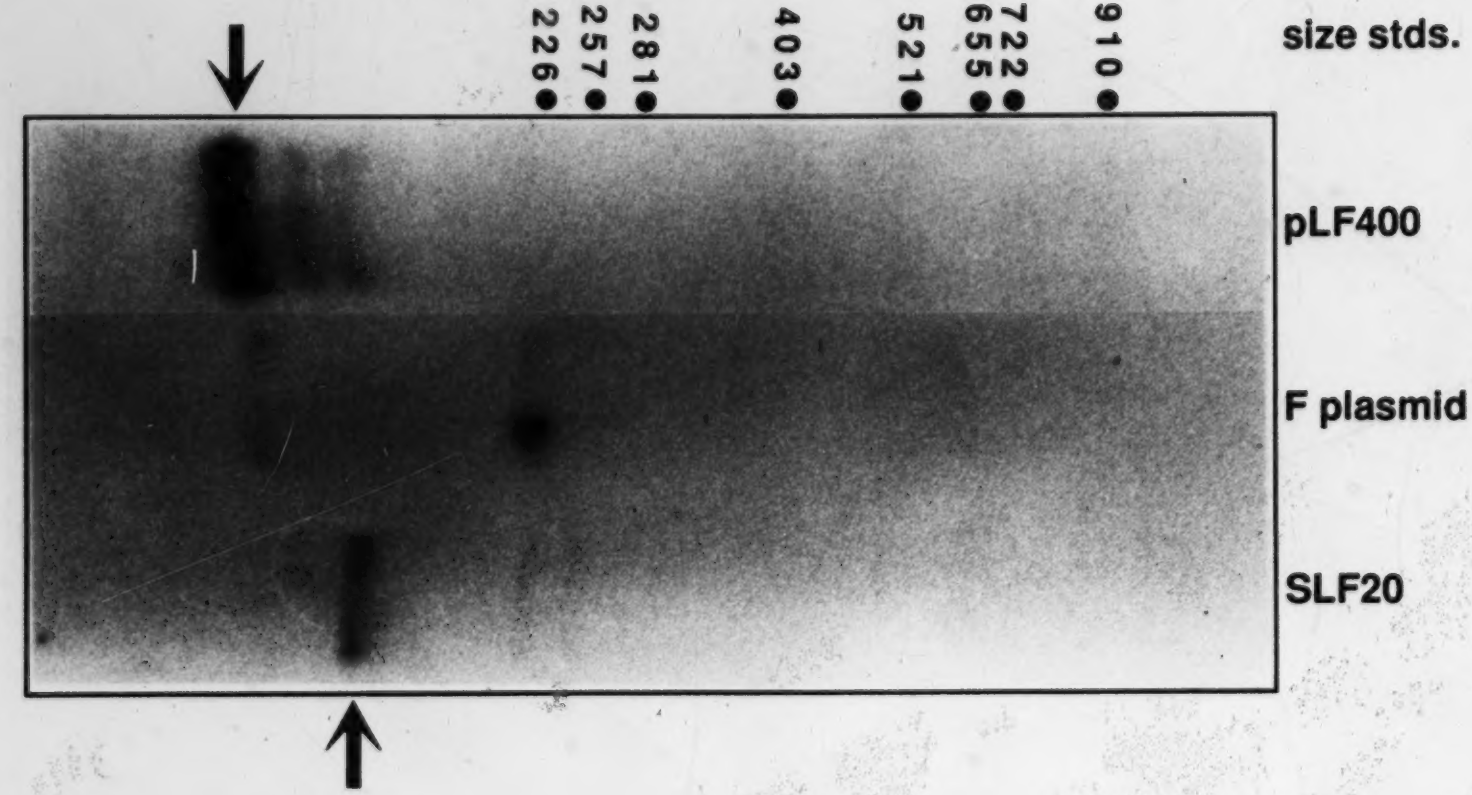

LEGEND
-finO bearing multicopy plasmid
-multicopy plasmid carrying cloned PfinP- mutation
- F pilus-specific phage
-F plasmid
F+ cells
recipient cells
CONJUGATION
mutant F-plasmid mates in finO's presence
Recombination with mutation in F-DNA cloned on multicopy plasmid
Phage sensitive Population in the presence of finO

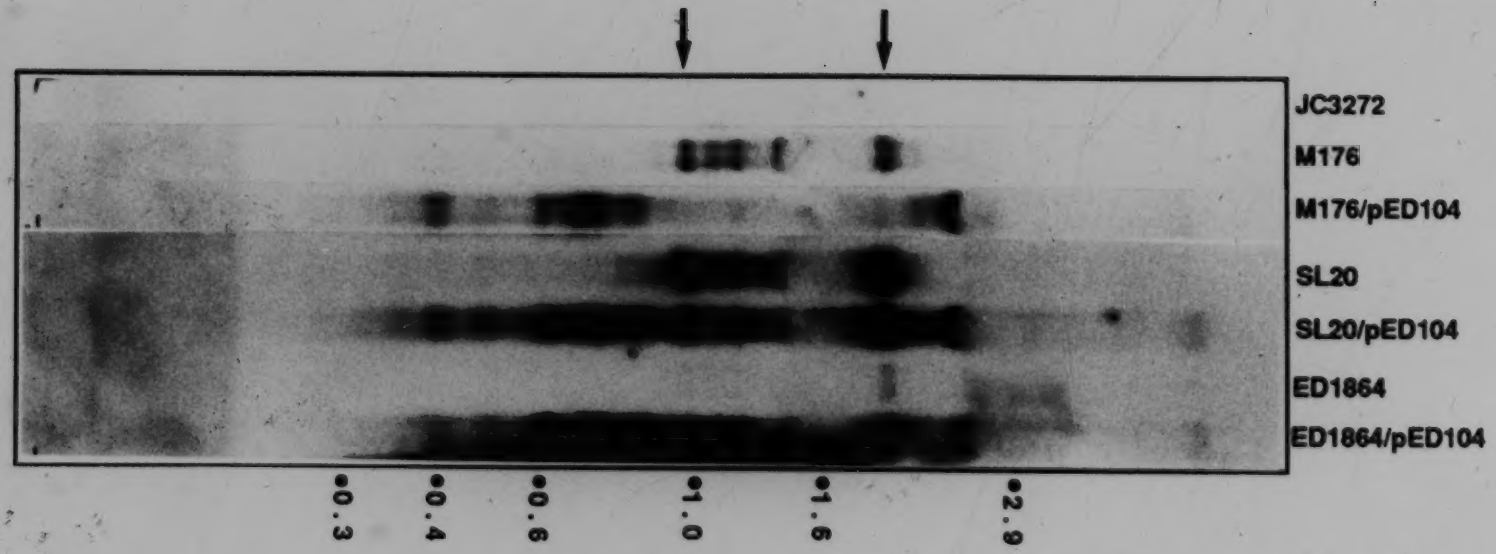
Figure III.7 Demonstration that the P_{finP} mutation had recombined into the F plasmid. This figure is an autoradiogram of a Southern blot of DNA samples digested with *Sau* 3A and *Rsa*I, and probed for *finP*. The probe was an oligonucleotide complementary to 15 bases in the 5' region of *finP*, radioactively labelled at its 5' end with ^{32}P. The arrows indicate the bands of interest - all larger bands are partial digestions. Dots represent DNA size standards. The DNA is pBR322 digested with *Alu* I; sizes are given in bases.


size stds.
910
722
655
521
403
281
257
226
pLF400
F plasmid
SLF20

merely an experimental artifact. This question was addressed in a number of ways: mating assays were performed to determine whether it mated any better than the F plasmid, electron micrographs were taken of SL20 cells to determine if they produced any more pili than F plasmid bearing cells, and Northern blots were performed, to see if there was, indeed, any more *traJ* mRNA produced in SL20 than in M176 (JC3272:JCFL0, an isogenic, wild-type F plasmid bearing strain).

The electron micrographs showed that SL20 did not produce any more pili than M176 (data not shown). Mating assays demonstrated that SL20 did not mate any better than M176. But Northern blots, probed for both the 5' and 3' end of *traJ*, demonstrated that there was significantly more *traJ* mRNA in the absence of the *finP* promoter. The comparison with other F plasmids was taken one step further by comparing RNA extracted from SL20 to that extracted from ED1864, a strain containing the F plasmid with the *fisO* mutation in *finP*. This strain has an active *finP* promoter, but an inactive antisense RNA molecule. Figure III.8 is an autoradiogram of a Northern blot of the RNA from the three strains, with and without *finO*. Clearly there is more *traJ* mRNA in the SL20 samples than in the other two. The patterns of the mRNAs do not change between the three strains in the absence of *finO*. Therefore, FinP is not acting to a significant degree to aid in the degradation of the *traJ* message. In the presence of *finO*, the band pattern of *traJ* mRNAs produced by ED1864 is the same as that of SL20 in the presence of *finO*. Since SL20 has no FinP, and therefore cannot produce a FinOP complex, this similarity suggests that the *fisO* mutation renders the FinO:FisP complex impotent. The origin of the smaller bands in strains containing cloned *finO* cannot be reliably determined, so there can be no comment on them, but they are probably not *traJ* RNA degraded by FinOP, because they appear in SL20/pED104, which has no FinOP. Densintometric traces of 3 replicate samples of RNA extracted from the three strains, Northern blottted, and probed for the 3' region of the *traJ* message showed that the ratio of detectable signal between the three plasmids was M176:ED1864:SL20-1:2.75:4.3. There was considerable sample to sample variation, but

Figure III.8 Comparison of *traJ* levels in M176, SL20, and ED1864. This figure is an autoradiogram of a Northern blot of total RNA from the strains indicated, probed for *traJ* mRNA. The probe is an internally ^{32}P-labelled, *in vitro* transcribed RNA transcript complementary to the 200 3' bases of the traJ message. Equivalent amounts or RNA, approximately 25 μg, have been added to each lane, with the exception of the strain ED1864, which contained 29% of the amount allocated to the others, and ED1864/pED104, which contained 87% of the amount allocated to the others. The position of RNA size standards are indicated by dots, their sizes given in kilobases. The arrows point to *traJ* transcripts discussed in the text. Bands between the two species are taken to be degradation products of the largest band.


JC3272
M176
M176/pED104
SL20
SL20/pED104
ED1864
ED1864/pED104
0.3
0.4
0.6
1.0
1.6
2.9

in no case did the F plasmid produce more *traJ* mRNA than ED1864, which, in turn, never produced more mRNA than SL20. This finding demonstrates that in the complete absence of *finO*, there is considerable repression of *traJ* expression by both unaided FinP and the opposing *finP* promoter, though this repression is not as dramatic as that seen in the experiments involving the induction of cloned *traJ*. It also shows that the expression of *traJ* is not the limiting factor in F plasmid transfer because despite the excess of *traJ* message, transfer functions are not summarily increased.

C. Discussion

The finding that FinOP reduced the amount of detectable *traJ* mRNA was not surprising. In Figure III.1 it was noted that there were two major bands hybridizing to anti-*traJ* probes; one of a size of about 2000 bases and the second of about 950 bases. The larger transcript obviously carries more than just the *traJ* message on it - this represents read-through transcription to *traY*, because probes for *traM* RNA did not hybridize with it. Although the amount of the larger transcripts recognized by the *traJ* mRNA probe decreased when *finO* was added to F plasmid-containing cells, as noted in Figure III.1, lane 3, a number of new, smaller bands appeared. The origin of these transcripts could be degraded *traJ* or could be from the vector pACYC177, since, in the absence of the F plasmid, this vector produces transcripts recognizable by an anti-*traJ* riboprobe. The present data support the accepted notion that FinOP acts to reduce the amount of *traJ* mRNA in cells (Willetts, 1977).

The successful cloning of *traJ* under the control of the powerful *lac* promoter demonstrated that if the expression of the gene can be controlled, an otherwise unstable construct can be made stable and easily maintainable.

The experiments involving the induction of expression of *traJ* from the *lac* promoter of pUC 19 provided some striking results, shown in Figure III.5. When the transcription of pSJ99 was induced with IPTG, the amount of mRNA transcribed was reduced at least 100-fold compared the the amount transcribed when pSJ88 was induced. The presence of

finP reduced the expression of *traJ* from the *lac* promoter. There was no accumulation of truncated RNA species representing *lacZ':traJ* RNA after twenty minutes. This observation argues against the first model presented in figure III.4, which predicted an accumulation of prematurely terminated RNA species. The second model in Figure III.4 has FinOP altering the pattern of RNA degradation of the *traJ* message. The results presented in Figure III.5 argue against this model because there is no significant difference between the pattern of the incomplete *traJ* RNA species between strains containing pSJ88 (*finP*$^-$) and pSJ99 (*finP*$^+$), which indicates that *traJ* RNA is being processed in the same manner in the presence and absence of FinOP. Therefore, it seems that FinP, unlike most common antisense RNA's, seems to act before the transcription of *traJ* begins.

It is difficult to imagine how a promoter or an antisense transcript could control the transcription of a powerful promoter that is at least 50 bases upstream of the termination of the transcript. It is possible that the effect observed resulted from a topological artifact introduced by cloning the fragment into pUC, which has many points of difference with the F plasmid. It is a small, 2.8 kb plasmid having a high copy number. Moreover, it is highly supercoiled, and is not attached to the cellular membrane nor to the chromosome. The F plasmid, on the other hand is 100 kb in size, is present at the level of one copy per chromosome, is attached to both the membrane and the host chromosome, and presumably has DNA topology more in keeping with that of the chromosome (Willetts, Skurray, 1987). The possibility exists that truncated *traJ* RNA fragments produced by FinOP activity have a half-life too short to be detectable by Northern blotting. This possibility limits any interpretation of the experiment. Another point worth considering is that through the P_{finP} site-specific mutation, a subtle change in the overall structure of *traJ* RNA was introduced, and this caused it to be much more labile. At the present time, it is not possible to quantitate the amount of FinO in the cell, and thus the stoichiometry of the FinOP system vs. the amounts of induced *traJ* mRNA is uncertain. Lastly, the strength of the *finP* promoter is unknown. According to Mullineaux (Mullineaux, Willetts, 1985), its strength

is about 1% that of P_{traJ}, but that value has been challenged by Dempsey, who argues that this value is a gross underestimation of its true strength (W. Dempsey, pers. comm.). These unknown variables will have to be determined before the results of the *traJ* induction experiments can be interpreted unambiguously.

Although there was extensive repression of *traJ* expression, when Northern blots containing RNA isolated from *traJ* induction experiments were probed for FinP, there was no significant change in the amount of detectable FinP in the cell. This is in marked contrast to other systems (Krinke, Wulff, 1987; Case *et al.*, 1990b), where an increase in the transcription of the target sequence results in a decrease in the concentration of the antisense RNA, as the RNA duplexes formed by the interaction of the species are degraded by host nucleases.

To overcome these problems, and to place the results in a more familiar experimental context, the mutation that eliminated *finP* promoter activity was recombined into the F plasmid. The only detectable difference between the transcripts produced by this site-specific mutant and those produced in cells containing the wild type F plasmid was seen on Northern blots. Though the profiles of the mRNA species were the same in both cases, there was a greater abundance of *traJ* mRNA produced by cells containing the site-specific mutant - at least 4-fold. By comparing the mRNA levels between the wild type M176, the *fisP* mutant ED1864, which has a wild-type promoter but a non-functional FinP, and SL20, which does not have a *finP* promoter, it seemed that the contribution of the promoter and the RNA molecule to the reduction of *traJ* expression were approximately the same. Experiment to experiment variation precluded any strong indication of a difference; in some cases SL20 produced >10X more *traJ* mRNA than M176, and in some cases only about 2X, but always the order of intensity of *traJ* bands was M176<ED1864<SL20. Therefore it was concluded that in the absence of *finO*, *finP* effectively represses the expression of *traJ*, but this decrease in *traJ* expression is not sufficiently dramatic to lead to a decrease in transfer.

Though the reduction in *traJ* expression noted in pSJ99 was confirmed to occur in cells containing the F plasmid, we were unable to deduce the stage of transcription at which FinP repressed *traJ* expression. Presumably, plasmid transfer is maximal as long as the amount of *traJ* mRNA is above a critical threshold level.

For the first time, the role of FinP on its own as an antisense RNA has been addressed and it was determined that both the act of *finP* transcription from the *finP* promoter and FinP itself contribute to a significant reduction in *traJ* mRNA levels. These results support the hypothesis of FinOP acting at the transcriptional level, and also bring to light yet another level at which *traJ* is controlled; through the activity of FinP. This presumably happens through promoter interference, and inefficient antisense activity by FinP. Thus FinO can be looked upon as a molecule that enhances the activity of FinP.

Chapter IV. The Effect of *finO* on FinP

A. Introduction

FinOP is unique among antisense RNA repression systems because two gene products are required for the system to perform its function. The role played by FinP can be understood through traditional models of antisense RNA action (Tomizawa, 1987; Kleckner *et al.*, 1987; Liao, McClure, 1988), which involve interaction between the loops of the antisense RNA and the target, followed by duplex formation between the two molecules. This occludes the ribosome binding site of the mRNA, and inhibits the target gene's expression (Ma, Simons, 1990). But what role does FinO play in this scenario?

Assuming that FinO is a protein, the only parallel system known is that of the ColE1 copy number control system, in which the Rom protein recognizes the complex formed between the RNA molecules in the initial stages of their interaction ('kissing') (Tomizawa, 1987; Eguchi, Tomizawa, 1990; Liao, McClure, 1988), binds to the complex, encourages duplex formation and then dissociates from the duplex (Eguchi, Tomizawa, 1990; Liao, McClure, 1988). Dempsey has shown that the presence of *finO* in cells causes a marked increase in the amount of R100 and F plasmid *finP* RNA found in cells (Dempsey, 1987), and Koraimann et al. has shown the same for the R1 plasmid (Koraimann *et al.*, 1991). This suggests that the function of FinO would be to increase the

amount of FinP through increasing *finP* transcription or FinP stability. These functions are not consistent with a model based on Rom activity, unless the increased FinP levels are due to the association of FinP with *traJ* RNA in a duplex that is resistant to cellular RNases (Dempsey, 1987; Frost *et al.*, 1989). In that case, the increase in FinP would be associated with *finO* presence because FinO makes duplex formation between FinP and *traJ* RNA more efficient. The role that FinO plays in the FinOP repression of *traJ* is not clear, and there are a number of possible modes of action that must be distinguished between.

This chapter seeks to discriminate between these possibilities for FinO function. First, it was confirmed that in cells containing the F plasmid complemented with an R6-5 *finO* gene, the observed amount of FinP was increased. In addition, evidence is presented indicating that the presence of *finO* partially compensates for mutations in FinP that reduce its stability and its abundance in cells. Finally, the question of the effect of *finO* on the stability of FinP is approached directly by producing FinP from an exogenous promoter in the presence and absence of *finO*. It is shown that the presence of *finO* is associated with a significant increase in the stability of FinP from a half-life of about 7.5 minutes to greater than 40 minutes. This increase is observed in the absence of *traJ* transcripts, suggesting that FinO interacts directly with FinP in a manner that protects the RNA from endonucleolysis.

B. Results

B.1 Examination of *finP* mutants' RNA

FinP was detected as a single RNA species of an apparent size of approximately 80 bases (Fig. IV.1). This result has always been obtained, regardless of the method used to denature the RNA; glyoxal treatment, formamide/formaldehyde, and urea have all been used, and in all instances, FinP has been detected at a size of about 80 bases, running on the gels among the tRNA. It was also shown that, like R100 (Dempsey, 1987) and R1 (Koraimann *et al.*, 1991) FinP, the amount of F plasmid FinP increased substantially in the presence of *finO* (Dempsey, 1987). The cause of this increase was unknown, and it is this question which is addressed in the following experiments.

In the publication of Frost et al. (Frost *et al.*, 1989), a number of mutant FinP molecules were examined. Some conferred the *finP*⁻ phenotype described by Finnegan (Finnegan, Willetts, 1971), and some the semi-dominant *fisO* phenotype. The mutant genes were cloned into pUC18 on the same *BglII* fragment as pNY300 (see Methods), and were sequenced. The sequencing data showed that all the mutations were base changes in the stem region of FinP (Fig.IV.2) (Frost *et al.*, 1989) that disrupted the base pairing of the complementary strands of the stem and led to a loss in the stem's free energy, making it less stable. An additional mutation that also disrupted base-pairing in stem I, 300A, was introduced by site-specific mutagenesis. A mutation that introduced a base-pair into stem I, pNY300B, was also created through site-specific mutagenesis. All the constructs were cloned with the *finP* promoter promoting transcription in the same direction as the *lac* promoter of pUC. Therefore, each construct had the potential to produce two transcripts, one of 80 bases, the wild-type FinP, and one of 180 bases, a chimeric transcript containing both *lacZ'* mRNA and *finP* RNA.

Strains were constructed containing the F plasmid and multicopy chimeras carrying the mutant genes with and without a third plasmid containing *finO*. RNA was extracted from these strains, and blots of the RNA were probed for FinP (Fig.IV.3). In the absence of

Figure IV.1 Autoradiogram of a Northern blot probed for FinP showing increased FinP in the presence of *finO*. The probe was an oligonucleotide complementary to the 12 5' bases of FinP, prPa, labelled at its 5' terminus with ^{32}P. 20 μg of RNA were added per lane, the RNA was separated on an 8% denaturing PAG, and transferred electrophoretically to a Zeta-Probe membrane.

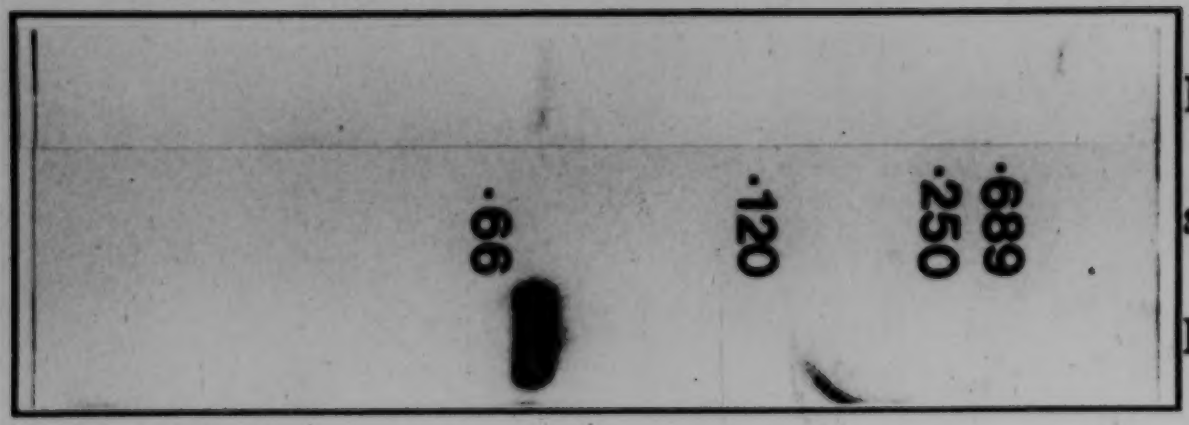
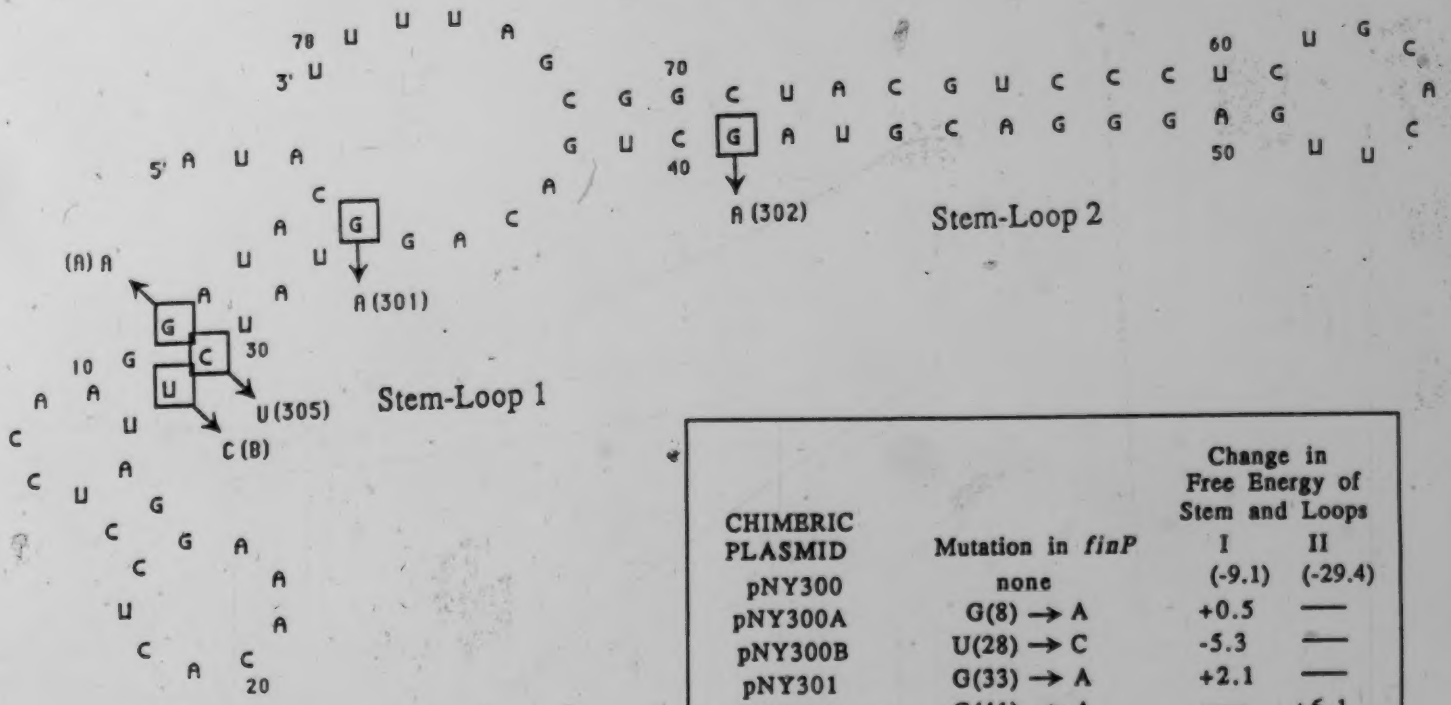

M176
size stds.
M176/pED104
.689
.250
.120
.99

Figure IV.2 A diagram of FinP showing the mutations that were studied. Each base altered by a mutation is boxed. An arrow points to the corresponding base found in the mutant gene, and the number or letter in parentheses denotes the name of the mutation. In the case of mutations (A) and (B), which were created by site-specific mutagenesis, they are named by appending the letters onto the plasmid that was used as a template for the mutagenesis, eg.pNY305A. The boxed table indicates the effect of the mutations by showing the change in free energy of the stem in FinP that is affected. A positive value indicates a destabilizing effect, and a negative value indicates a stabilizing effect. The information for this table is from Frost et al. [6].



CHIMERIC PLASMID	Mutation in *finP*	Change in Free Energy of Stem and Loops I (-9.1)	II (-29.4)
pNY300	none		
pNY300A	G(8) → A	+0.5	—
pNY300B	U(28) → C	-5.3	—
pNY301	G(33) → A	+2.1	—
pNY302	G(41) → A	—	+6.1
pNY305	C(29) → U	+2.8	—
pNY305A	C(29) → U U(28) → C	+0.5	—

Figure IV.3 The effect of *finO* on the abundance of mutant FinP molecules whose genes have been cloned into pUC18. This figure is an autoradiogram of a blot probed for FinP. The host strain is M176, with and without pED104, as indicated. Each strain also contains a pUC/F plasmid chimera, as described in the text. These chimeras are indicated on the figure. 10 μg of total RNA was run on each lane, and the RNA was separated on a formaldehyde/2% agarose gel. The RNA was electrophoretically transferred to a Zeta-Probe membrane, and probed with oligonucleotide prPa, labelled at its 5' terminus with ^{32}P.

+JCFLO
+JCFLO
+pED104
pNY300
pNY300A
pNY300B
pNY301
pNY305
pNY305A
Standards
pNY300
pNY300A
pNY300B
pNY301
pNY302
pNY305
pNY305A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
689
250
66

finO, probed blots had to be overexposed to demonstrate the presence of RNA carrying the stem-destabilizing mutations pNY301, 305, and 305A. pNY300A had very little of the 80 base FinP species, though there was abundant chimeric transcript. These findings were consistent with published studies on mutant antisense RNA molecules, which reported a dramatic decrease in the amount of RNA when mutations that ruin the complementarity of paired bases in its stem are introduced (Case *et al.*, 1990a). This decrease was reported to be proportional to the magnitude of the loss in stability of the stem's duplex structure conferred by the mismatch (Case *et al.*, 1990a). Since all the *finP* mutations mentioned decreased the stability of stem I, the decrease in their abundance can be attributed to a decrease in stability. pNY300B, which contained a base change that introduced a base pair into the stem, produced more *finP* RNA than the wild-type gene cloned on pNY300. This, too is consistent with results published by Case et al. (Case *et al.*, 1990a), where mutants that had more stable stems accumulated to a greater level than the wild-type transcript in the cell.

In the presence of *finO*, there was abundant FinP RNA in all samples. In the case of the mutants pNY301 and pNY305, only the amount of the chimeric transcript was increased, but pNY300A and pNY305A showed increases in the amounts of both the chimeric and the FinP transcript. pNY300 and pNY300B both produced more *finP* RNA in the presence of *finO* than its absence.

B.2 Induction of *finP* Expression in the Presence and Absence of *finO*

The observation that *finO* could increase the amount of otherwise rare transcripts originating from an exogenous promoter suggested that finO was acting at the post-transcriptional level and led us to propose the following experiment. *finP* was cloned as a *Sau*3A - *Rsa*I fragment (see Methods) that contained the entire structural gene for *finP* but neither its promoter nor *traJ*'s promoter. The fragment was cloned into the vector pTTQ18 which has an IPTG inducible *tac* promoter, and carries the *lacI* gene with the *lacI*^{q} mutation

which can produce enough LacI to repress the *tac* promoter. Cells carrying this construct in the presence and absence of *finO* were grown to an OD_{600} of 0.35-0.5 and induced with IPTG for one minute. Then rifampicin was added to stop any further transcription of the gene and timed samples were removed at appropriate intervals. RNA was extracted, run on a denaturing polyacylamide gel, blotted and probed for FinP as above.

The results in Figures IV.4 and IV.5 represent typical experiments. The upper band on the Northern blot is a chimeric transcript resulting from transcription initiating at P_{tac}, the lower band is the stable FinP molecule. The upper band's half-life seems independant of *finO*; it disappears within 5 minutes. The FinP molecule, however, does respond to *finO*. In the absence of *finO*, the half life of FinP is about 7 minutes, as determined by counting the radioactivity in the bands that exposed the X-ray film. In the presence of *finO*, its half life was found to be greater than 40 minutes, the generation time of the culture. In this experiment, *finO* is associated with an increase in the stability of FinP. This result was consistent with the fact that it was also associated with an increase in the amount of unstable mutant FinP molecules. On the basis of the results, it was concluded that *finO* causes the increase in FinP concentration through a stabilization of the transcript.

Figure IV.4 Induction of *finP* in the absence of *finO*. *finP* was induced through the addition of IPTG to early log phase cells containing pSQ150. Rifampicin was added one minute after IPTG addition. Samples were removed at timed intervals following rifampicin addition. The intervals as indicated are in minutes. 10 μg of total RNA was extracted and electrophoresed on an 8% denaturing PAG. The RNA was electrophoretically transferred to Zeta-Probe membrane, and probed with the oligonucleotide prPa labelled at its 5' terminus with ^{32}P.

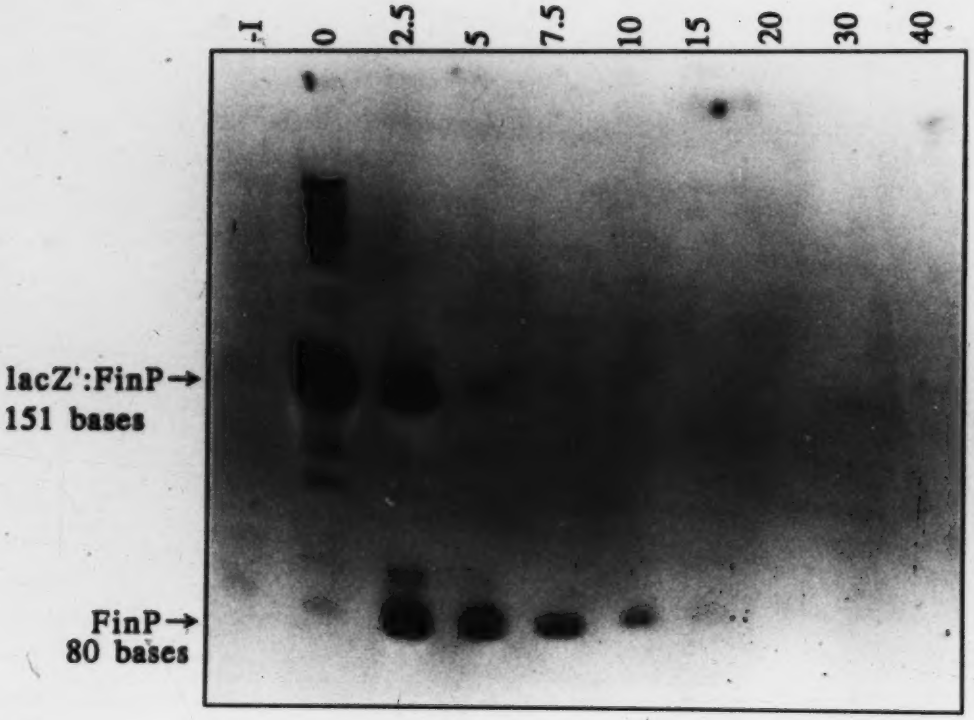
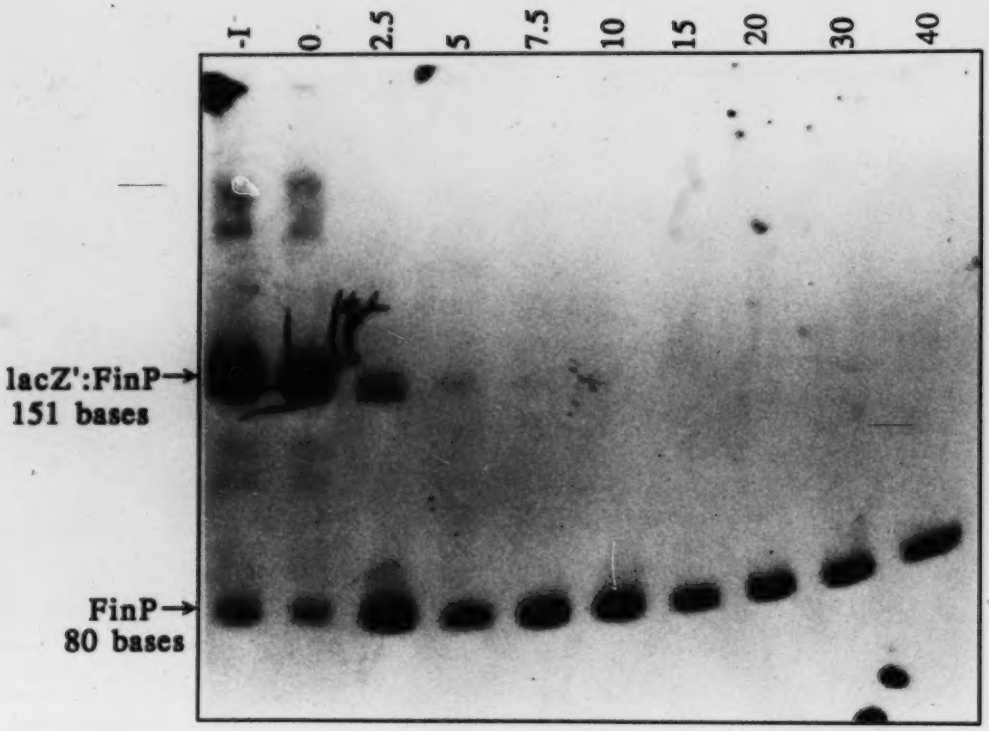

-1
0
2.5
5
7.5
10
15
20
30
40
lacZ':FinP→
151 bases
FinP→
80 bases

Figure IV.5 Induction of *finP* in the presence of *finO*. *finP* was induced through the addition of IPTG to early log phase cells containing pSQ150. Rifampicin was added one minute after IPTG addition. Samples were removed at timed intervals following rifampicin addition. The intervals as indicated are in minutes. 10 μg of total RNA was extracted and electrophoresed on an 8% denaturing PAG. The RNA was electrophoretically transferred to Zeta-Probe membrane, and probed with the oligonucleotide prPa labelled at its 5' terminus with ^{32}P. In this particular experiment, the before induction and t=0 samples were mixed together; subsequent experiments showed that significant expression of *finP* occurred only in the presence of IPTG.


-1
0
2.5
5
7.5
10
15
20
30
40
lacZ':FinP→
151 bases
FinP→
80 bases

C. Discussion

The effect of *finO* upon the amount of FinP in a cell had been noted before (Dempsey, 1987). The purpose of this chapter was to gain an understanding of how the *finO* gene product causes an increase in the concentration of FinP in the cell.

The first clue to the mechanism was derived from hybridization experiments of Northern blots containing total RNA extracted from cells carrying the F plasmid and recombinant plasmids carrying DNA fragments with point mutations in *finP*. It had been shown previously (Case *et al.*, 1990a) that the antisense RNA, RNA-OUT of the insertion sequence IS10 is a very stable molecule with a half-life of about 60 minutes, and its ability to repress *tnp* expression was dependent on that stability. Mutants with mispairings in the duplex stem of the antisense RNA produced less RNA and repressed much more poorly that wild-type molecules. Similarly, as seen in Figure IV.3, *finP*⁻ mutants produced much less detectable RNA than the wild-type - even when transcribed from the *lac* promoter of pUC. The mutations decreased the mutant molecules' stability, and lead to lowered levels of FinP RNA. For reasons which are presently unclear, pNY300A seemed to be an exception to this rule on this particular blot.

The introduction of a *finO* gene to the cells caused an increase in the amounts of all types of FinP molecules, but most dramatically those containing mutations. This result argues that FinO is acting at the post-transcriptional level to stabilize the mutant transcripts whose stability was reduced by the point mutations. It is unlikely that FinO would act at the transcriptional level, increasing the transcription of both the *lac* and *finP* promoters because most transcriptional activators are specific for the promoters that they affect.

The possibility of FinO acting post-transcriptionally was tested directly by the induction experiments. They clearly showed that FinO increased the half-life of FinP indefinitely, from 7 minutes to longer than a cell generation time.

Because the cloned *finP* gene has no *traJ* promoter, and no F plasmid is in the cell, there is virtually no *traJ* mRNA in the cell (see Chpt.V, pSQ150 probed for *traJ* mRNA).

Therefore the stabilization of FinP is not linked to its interaction with *traJ* mRNA. FinO cannot be considered a protein with a function analogous to Rom, since Rom recognizes the complex between two RNA molecules (Eguchi, Tomizawa, 1990). This also eliminates the possibility that the increase in FinP was due to its formation of a stable duplex with *traJ* mRNA, as proposed by Dempsey (Dempsey, 1987) and Frost (Frost *et al.*, 1989). This finding also argues against the notion that FinO is an RNA complementary to a region of *traJ* mRNA. Dempsey proposed (Dempsey, 1987) that the binding of this *finO* RNA would force *traJ* mRNA into a configuration recognizable by FinP, and thereby enhance the repression reaction. In this case, FinO has stabilized FinP in the absense of the *traJ* mRNA that it interacts with.

What type of model do the data support? We have shown that in the absence of *traJ* mRNA, *finO* increased the half-life of *finP* RNA. But it is well known that this increase in FinP concentration due its stabilization by FinO occurs in the presence of *traJ* mRNA also (Dempsey, 1987; Frost *et al.*, 1989; Koraimann *et al.*, 1991). Thus the stabilizing affect of *finO* upon FinP occurs both in the presence and absence of the repression reaction. A logical extrapolation would be that since it happens regardless of repression, the association occurs independently of the FinOP *traJ* mRNA interaction. The data presented therefore support the notion that FinOP exist in the cell as a ribonucleoprotein (RNP) and repress *traJ* expression in a reaction secondary to their association.

Chapter V. Characterization of Transcripts Participating in Regulation of Transfer

A Introduction

It is generally believed that the regulation of the transfer of F-like plasmids involves the interaction of the transcripts of the *finP* and *traJ* genes, with FinO participating in some uncharacterized manner to increase the efficiency of the interaction (Willetts, Skurray, 1987). This notion was challenged by Dempsey (Dempsey, 1989) while investigating the transcripts of the *traM* gene of the F-like plasmid R100. The *traM* gene is directly upstream of *traJ*, and is transcribed in the same direction (Willetts, Skurray, 1987). The function of its gene product is not known, but it is thought to be a signal for the commencement of conjugation (Willetts, Skurray, 1987). In support of this idea, TraM of the F plasmid binds to the *oriT* region (L. DiLaurenzio, pers. comm.). Dempsey determined, using RNase protection assays to map the 5' end of the *traM* transcripts, and Northern blots to size them, that the mRNA produced by the *traM* gene must terminate within *traJ*, within a DNA sequence that is complementary to the *finP* promoter (Dempsey, 1989). Therefore mRNA originating at *traM* would contain sequences capable of hybridizing with *finP* RNA. Dempsey proposed that these *traM* transcripts would reduce the amount of FinP available in the cell to repress *traJ* by hybridizing with the FinP, and thus function to alleviate the

FinOP repression of *traJ*. If this result were true for the F plasmid, it would add another variable to those already known that affect the frequency of F plasmid conjugation.

The question that Dempsey's discovery prompted us to ask was 'do trancripts originating from the *traM* promoter contain sequences that could hybridize with FinP in a way that affects F plasmid transfer?' To answer this question we constructed a series of six recombinant plasmids, each one carrying a different combination of the promoters for *traM*, *traJ* and *finP*. The ability of these cloned DNA fragments to influence F plasmid DNA transfer was determined through mating assays, and Northern blotting determinations with radioactively labelled oligonucleotides specific for *traM*, *traJ* and *finP* RNA. The rationale behind these experiments was based on the idea that if RNA transcripts from *traM* were able to hybridize with FinP, then the clone containing *traM* would derepress F plasmid transfer because the gene dosage of the cloned DNA would be greater than that of the *finP* encoded by the F plasmid. The majority of the FinP molecules encoded by F would interact with the RNA encoded by the cloned F plasmid DNA, not with F plasmid *traJ* RNA. The experiments reported in this chapter were designed to test this hypothesis by analyzing total RNA from the strains through gel electrophoresis, Northern blotting and probing the blots for the RNA sequences of interest. The results showed that in the cloned F plasmid DNA, only *traJ* produces a detectable transcript complementary to FinP, and it was concluded that *traM* RNA does not interfere with the FinOP repression system.

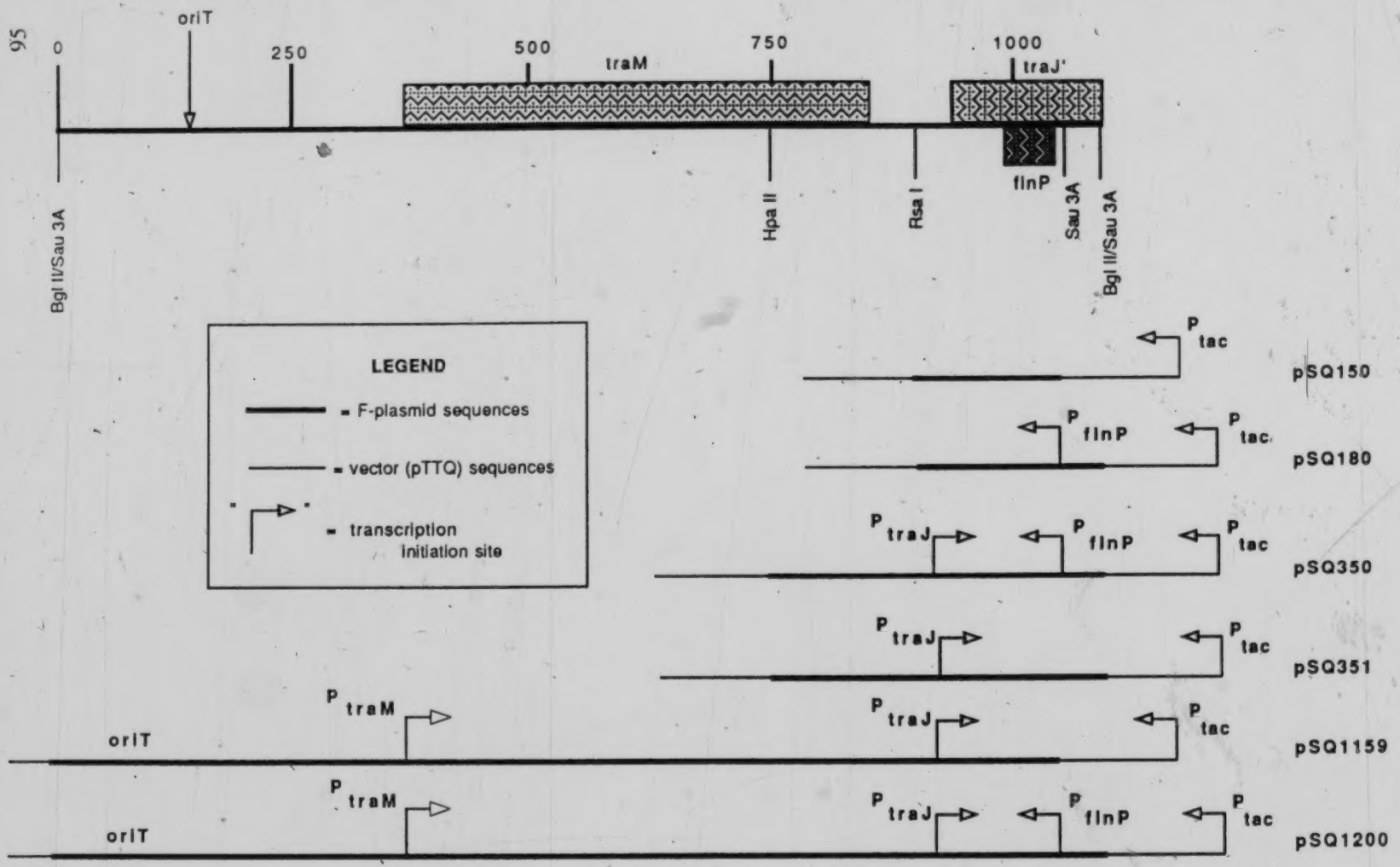
B. Results

B.1 Creating the Constructs

By cloning the portion of F plasmid DNA containing the sequences of interest into a multicopy vector, we hoped to create a sensitive assay for any transcript that would decrease FinOP activity through sequestering FinP in a non FinP:*traJ* mRNA duplex. In a background strain harbouring the F plasmid and *finO*, if any of the cloned genes produced RNA molecules that bound FinP, there would be a drastic reduction in the amount of free FinP, because the F plasmid could not produce enough FinP to compensate for the inundation of FinP-binding RNA molecules originating from the cloned genes. The reduction in unpaired FinP would reduce the amount of FinP available to hybridize with *traJ* RNA produced by the F plasmid and more *traJ* RNA would be translated, resulting in increased expression of the transfer operon, which would result in a higher transfer frequency in the population of F plasmid bearing cells.

Six clones were made, which were grouped into three pairs (see Fig.V.1). Each pair of constructs included one that did and one that did not produce FinP. Because the *finP* bearing fragment would be expected to produce enough FinP to compensate for any anti-FinP transcripts it produced, the fragment would serve as a control for any other effect the DNA fragment might have on the the regulation of transfer. For example, a fragment containing the origin of transfer could repress F plasmid transfer through sequestering the *oriT* binding proteins in an *oriT* complex that would lead to the transfer of the multicopy plasmid, not the F plasmid. The fragment that did not carry *finP* would serve as the test fragment because it could not produce any FinP to interact with the anti-FinP transcripts also produced. By comparing the mating frequencies of strains containing the pairs of clones with and without *finP*, conclusions could be drawn about the nature of the transcripts of the cloned fragments' interaction with FinP.

Figure V.1 A diagram of the clones used in the mating assays to determine the effects of upstream sequences on F plasmid transfer. The shaded boxed regions in the upper part of the diagram represent the transcribed portion of the genes. Restriction sites identified are those used to construct the different clones.


oriT
0
250
500
traM
750
1000
traJ'
finP
Bgl II/Sau 3A
Hpa II
Rsa I
Sau 3A
Bgl II/Sau 3A
LEGEND
= F-plasmid sequences
= vector (pTTQ) sequences
= transcription initiation site
P tac
pSQ150
P finP
P tac
pSQ180
P traJ
P finP
P tac
pSQ350
P traJ
P tac
pSQ351
P traM
oriT
P traJ
P tac
pSQ1159
P traM
oriT
P traJ
P finP
P tac
pSQ1200

The entire *traJ* gene was not present on any of these clones, but the sequences that hybridized with FinP were present. With only the 5' region of *traJ* (*traJ'*)on the clones, the experiments simply measured the effect of the interaction of the transcripts originating from the cloned DNA on the abundance of full-length *traJ* mRNA transcribed from the F plasmid.

Since the purpose of these experiments was to measure the effect of the cloned F plasmid fragments' transcripts on the repression system of F, it was important that the promoters on the vector not transcribe the cloned fragments. To ensure this, the vector chosen was pTTQ18/19 whose *tac* promoter was under the control of LacI produced by the *lacI*^{q} gene present on the vector.

pSQ1200 (Fig. V.1) contained the entire *Bgl*II fragment which codes for *oriT*, *traM*, *traJ'* and *finP*. The fragment cloned into pSQ1159 shared the 5' *Bgl*II boundary with pSQ1200, but it had a 30 base pair *Sau*3A fragment deleted at its 3' end. This fragment contained the *finP* promoter. Therefore this deletion mutant could not transcribe *finP*. pSQ1159 contained *oriT*, *traM* and *traJ'*.

The fragment cloned in pSQ350 and pSQ351 was bordered on the 5' side by the *Hpa*II site found in *traM* and by the *Bgl*II site in *traJ* at its 3' end. pSQ351 was a site-specific mutant of pSQ350, and carried the *finP* promoter-down mutation described in Chapter III. pSQ350, thus, contained *traJ'* and *finP*, and pSQ351 contained only *traJ'*.

pSQ180 contained the DNA fragment that codes for *finP*. Its 5' boundary was the *Rsa*I site found within the *traJ* promoter, and its 3' boundary was the *Bgl*II site within *traJ*. pSQ180 contained only *finP*. pSQ150 was also bounded on the 5' end by the *Rsa*I site, but its 3' end was the *Sau*3A site within the *finP* promoter. pSQ150 produced no transcript. It served as a multicopy plasmid control to ensure that the metabolic load of maintaining another plasmid does not interfere with F plasmid transfer or its regulation. It also served as a control for the effect of the DNA that contained the FinP binding site. One of the proposed possibilities of FinP activity was that FinP bound the DNA sequence that it

was complementary to. The effect of pSQ150 on the mating efficiency of the F plasmid might yield a clue as to whether the DNA-binding paradigm was valid.

B.2 Mating Assays

Each of the constructs described above was transformed into an *E. coli* strain containing the F plasmid JCFLO and *finO* cloned on the multicopy plasmid, pED104. The effect of the transcripts originating from the cloned F plasmid DNA in the regulation of F plasmid transfer was monitored through mating assays.

Mating assays were performed as described previously (Frost *et al.*, 1989). The host strain was *E.coli* M176 (JC3272/JCFLO).The mating frequency of this strain served as the standard to which all other strains' mating ability was compared. Other donor strains were M176/pED104 (*finO+*), and M176/pED104 with one of the pSQ plasmid constructs. The recipient strain was *E.coli* ED24. Mating frequency was expressed as transconjugants per 100 donors. The frequency was then expressed as a percentage of the mating frequency of M176. The results of these experiments are summarized in Table V.1.

M176/pED104/pSQ1200 mated at a level of 0.5% of the frequency of M176, about the same frequency as M176/pED104, which transferred at 1.0% of the frequency of M176. Thus the *finP* on the construct was able to compensate for whatever anti-FinP transcripts were being produced by *traJ* or *traM*, and the overall regulation of the F plasmid remained unchanged. This result also shows that the presence of multicopy *oriT* did not repress F plasmid transfer. Unfortunately, pSQ1200 picked up the same *finP* mutation as found in pNY301 (see fig.IV.2), and the results from the first mating could not be repeated. It is believed that the data presented is an accurate reflection of the effect of pSQ1200 on the mating efficiency of the F plasmid because in a mating assay done at the same time, it was able to repress the mating ability of SL20, which produces no FinP (see fig.III.5). Therefore the results from the mating assay quoted represent the effect of pSQ1200 with a wild-type *finP*. The addition of pSQ1159 to M176/pED104 cells resulted in an almost complete derepression of F plasmid mating ability, from 1% to 76% of M176

Table V.1 The effect of the clones diagrammed in fig V.1 on the mating efficiency of cells carrying the F plasmid and *finO*. M176 is JC3272/JCFLO. pED104 is the R6-5 *finO* cloned on a Pst I fragment in pACYC177.

TABLE V.1
The Effect of Exogenous F Plasmid Genes on The Mating Frequency of E.coli Strain M176/pED104 With E.coli Strain ED24

Strain	Promoters on Cloned Fragment	Mating Frequency	Range (at least 2 assays performed)
M176	N/A	100%	
M176/pED104	N/A	1.0%	0.4% - 2.4%
M176/pED104/pSQ150	none	1.3%	0.9% - 1.8%
M176/pED104/pSQ180	P_{finP}	0.03%	0.02% - 0.04%
M176/pED104/pSQ350	P_{finP}, P_{traJ}	40%	39% - 80%
M176/pED104/pSQ351	P_{traJ}	130%	35% - 281%
M176/pED104/pSQ1159	P_{traJ}, P_{traM}	76%	43.% -108.%
M176/pED104/pSQ1200	P_{finP}, P_{traJ}, P_{traM}	0.5%	0.5%

This table descibes the effect of the clones diagrammed in Fig.V.1 on the mating efficiency of cells carrying the F plasmid and *finO*. M176 is JC3272/JCFLO. pED104 is the R6-5 *finO* cloned on a *Pst*I fragment in pACYC177. "Px" is the promoter of gene x. Only one assay was performed on pSQ1200, as it lost the wild-type *finP* gene (see text).

frequency. Clearly, a transcript produced by the cloned F plasmid DNA on pSQ1159 interfered with the FinOP repression of F plasmid transfer.

Both pSQ350 and pSQ351 derepressed the transfer operon of M176/pED104 cells. M176/pED104/pSQ350 transferred at a frequency of 40% of M176 and M176/pED104/pSQ351 transferred at a frequency of 130% of M176. The fragment cloned in these plasmids contained *traJ'*, but no *traM*. This result argues against the participation of *traM* RNA in a derepression of F plasmid transfer.

pSQ180, a clone that contained only *finP*, repressed F plasmid transfer to 0.03% of M176. This represented a 30-fold increase in repression efficiency compared to M176/pED104 cells. The increased amount of FinP in the cells evidently causes FinOP repression to be more efficient. M176/pED104 cells containing pSQ150 transferred at 1.3% the frequency of M176, which is the same as M176/pED104 with no other plasmids present. Therefore the derepression observed in the strains containing pSQ1159, pSQ351 and pSQ351 was a consequence of the transcription of the cloned F plasmid fragments, and not a consequence of the cells having to respond to altered metabolic demands imposed by the multicopy plasmids.

B.3. Analysis of Transcripts

Total RNA was extracted from the strains described above and electrophoresed on an 8% denaturing polyacrylamide gel. The RNA preparations were then electrophoretically transferred to a Zeta-Probe membrane, and probed for sequences of interest with end-labelled oligonucleotides complementary to *finP*,*traJ*, and *traM* RNA. Transcripts from *traJ* and *finP* were detected, but none from *traM*. The results are shown in figures V.2 and V.3.

Both pSQ1200 and pSQ1159 produced *traJ'* mRNA. This transcript seemed to have been degraded by the host cell to the stable stem-loop structure that is the complement of FinP. This explained why there was no difference in the size of the RNA between clones that differed in length by 30 base pairs at the 3' end. pSQ1159 produced more *traJ'*

Figure V.2 The amounts of *traJ'* transcript present in the strains tested for mating ability. This figure is an autoradiograph of total RNA electrophoresed on an 8% denaturing polyacylamide gel, electroblotted to Zeta-Probe nylon membrane, and probed for *traJ* RNA species with an oligonucleotide complementary to bases 185 - 203 in the *traJ* mRNA, which had been radioactively labelled with ^{32}P. All lanes bearing 'pSQ' labels are RNA samples from M176/pED104 cells containing the plasmid indicated.

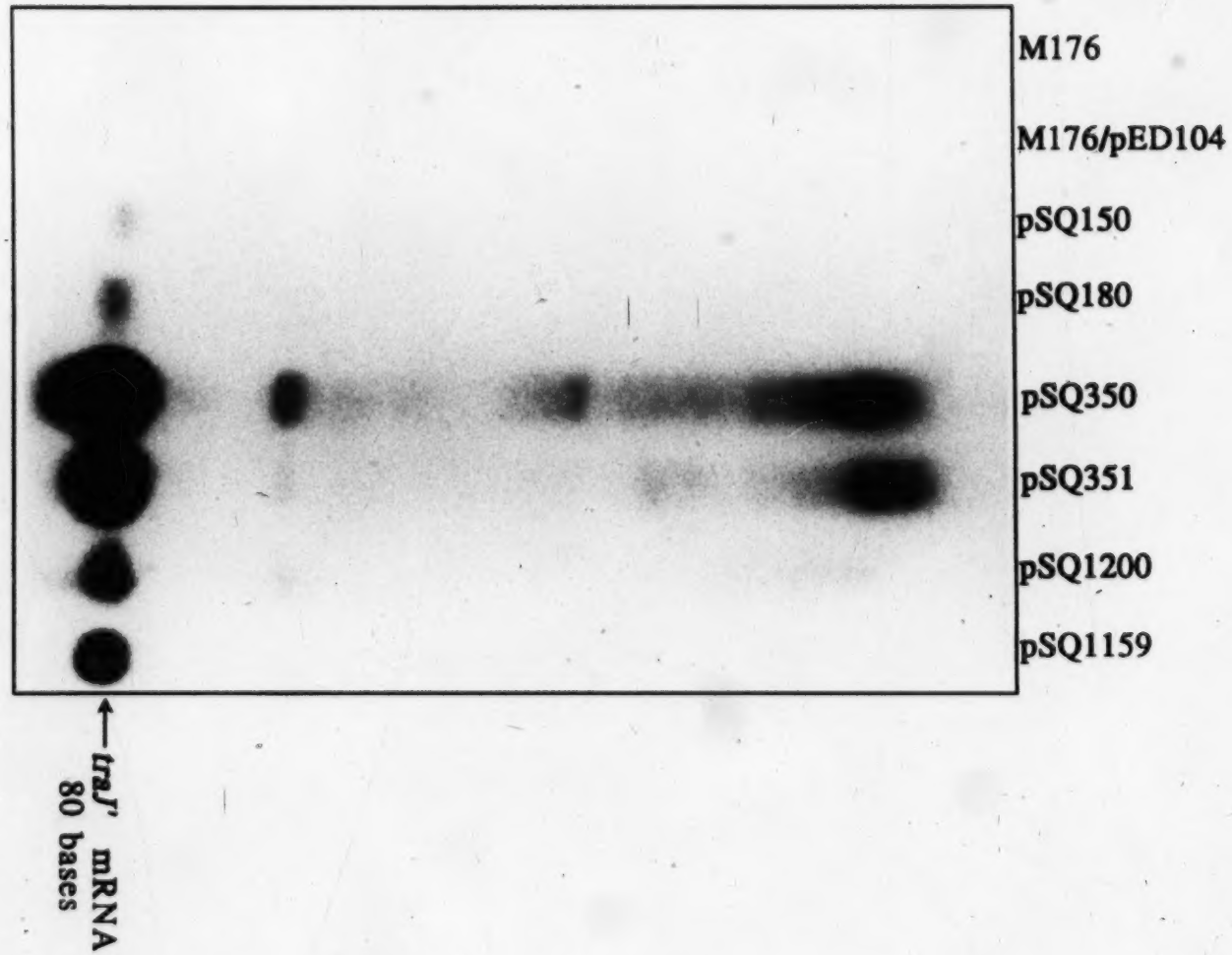
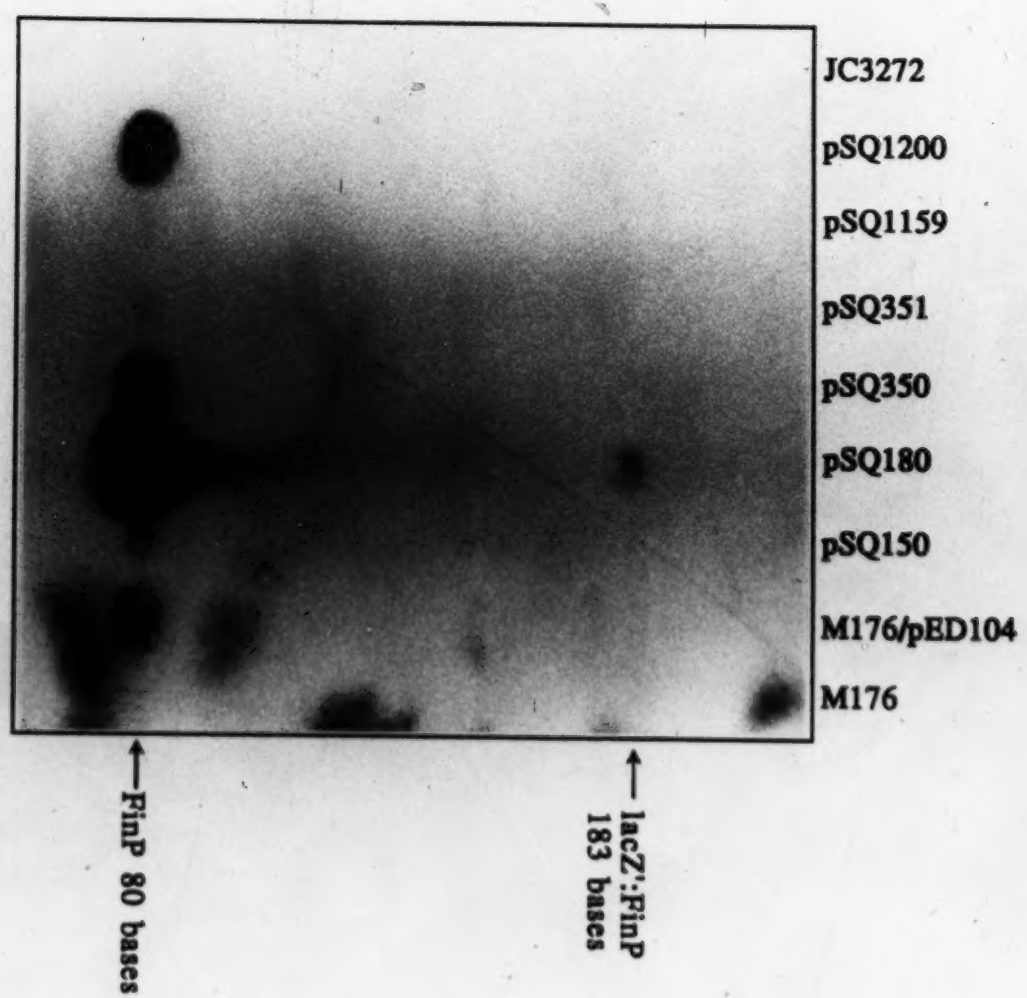

M176
M176/pED104
pSQ150
pSQ180
pSQ350
pSQ351
pSQ1200
pSQ1159
traJ' mRNA
80 bases

Figure V.3 The amounts of FinP present in the strains tested for mating ability. This figure is an autoradiograph of total RNA electrophoresed on an 8% denaturing polyacylamide gel, electroblotted to Zeta-Probe nylon membrane, and probed for FinP with the oligonucleotide prPa, which had been radioactively labelled with ^{32}P. All lanes bearing 'pSQ' labels are RNA samples from M176/pED104 cells containing the plasmid indicated.


JC3272
pSQ1200
pSQ1159
pSQ351
pSQ350
pSQ180
pSQ150
M176/pED104
M176
FinP 80 bases
lacZ':FinP
183 bases

RNA than pSQ1200, thus the derepression of F transfer observed in M176/pED104/pSQ1159 cells could be explained by the abundant *traJ'* transcript hybridizing with FinP transcribed from the F plasmid, lowering the amount of FinP in the cell available for *traJ* repression, and derepressing the F plasmid more efficiently than pSQ1200. The FinP transcribed from pSQ1200 presumably reduced the amount of free *traJ'* RNA originating from the clone and thus lessened its derepressive effect.

pSQ350 and pSQ351 produced the most *traJ'* mRNA. This observation is consistent with their increased ability to derepress M176/pED104 cells. Even though pSQ350 produced an appreciable amount of *finP* RNA, it was not enough to offset the derepressive effects of the *traJ'* RNA. It is presently not clear why the clones pSQ350 and pSQ351 produced much more *traJ'* RNA than pSQ1200 and pSQ1159. The basis for this difference is under investigation.

pSQ180 produced the most FinP of any of the clones, and very little *traJ'* RNA. This finding is consistent with its strong repressive ability because FinP is the proposed effector of repression, while *traJ'* RNA is a proposed derepressor. pSQ150 produced very little *finP* or *traJ* RNA. There was slightly less detectable FinP than in the background strain, and it contained the least amount of *traJ'* RNA of all the clones. This finding is consistent with the minor effect of pSQ150 on the repression of F plasmid transfer. It also demonstrated that the *tac* promoter was repressed very efficiently by LacI during the mating experiments.

B.4 IPTG Induction of pSQ351

It was proposed in the previous section that the derepressive effects of some clones were due to a molecular excess of *traJ'* RNA which titrated most of the FinP transcribed by the F plasmid by hybridizing with it, and allowing the F plasmid-borne *traJ* to be expressed. To test this hypothesis, we examined the effect of increasing the FinP concentration in cells carrying pSQ351, a derepressing clone. The host strain for these experiments was *E. coli* SL20, which is JC3272 carrying the F plasmid SLF20, which has

a mutation in the *finP* promoter that abolishes *finP* transcription. pED104 was added to provide *finO*. The complete strain was SL20/pED104/pSQ351. This strain was grown in the presence or absence of IPTG, and then mixed with *E.coli* ED24 to allow conjugative transfer. IPTG is an inducer of the *tac* promoter of pTTQ18, the vector for pSQ351, and its presence would result in the transcription of the cloned *finP* gene. If *traJ'* RNA was derepressing the F plasmid because it was present in molar excess to FinP, then the transcription of the *finP* gene by the *tac* promoter should alleviate the derepressive effects. The strain grown in the presence of IPTG transferred at an efficiency of 4.3 transconjugants/100 donors, which was 46-fold less than the strain grown in its absence, which mated at a frequency of 200 transconjugants/100 donors. Indeed, the increase of FinP concentration did act to repress transfer. Control experiments with SL20/pED104/pTTQ18 in the presence and absence of IPTG showed that the transcription from the *tac* promoter did not repress mating frequency. This observation provides the most direct evidence that the derepression of F transfer by specific clones was due to the titration of F plasmid FinP by *traJ'* RNA.

Additional evidence to support the idea that *traJ'* RNA caused derepression of F transfer was provided by a parallel experiment. pSQ150, which has no *traJ* or *finP* promoter, was transformed into SL20/pED104, and the resulting strain was mated with ED24 in the presence and absence of IPTG. The IPTG-induced strain transferred at an efficiency of 2.6×10^{-2} transconjugants/100 donors, which is approximately two orders of magnitude less efficient than the IPTG-induced strain containing pSQ351. Therefore, it was concluded that the active *traJ'* gene found on pSQ351 was responsible for the significant decrease in the effectiveness of FinOP at reducing the transfer efficiency of the F plasmid.

C. Discussion

In the discussion section of his paper, Dempsey (Dempsey, 1989) proposed that a multicopy plasmid bearing the *traM* and *traJ* promoters, but not the *finP* promoter could cause the derepression of a co-resident F-like plasmid, by flooding the cell with *traM* transcripts that hybridize with *finP* RNA. To test this hypothesis six constructs were prepared containing every possible combination of the *traM*, *traJ* and *finP* genes, with the exception of *traM* alone and *traM* and *finP*, both of which would require a mutation in the *traJ* promoter. These clones were transformed into M176/pED104 cells and tested for derepression of F transfer.

The background level of F transfer with M176/pED104 was found to be 1% of M176. When pSQ1200 was transformed into the cells, the frequency of mating remained unchanged. This is what was expected because pSQ1200 contained functional *traM*, *traJ* and *finP* genes. Therefore the dosage of the genes encoding these transcripts was equivalent, and there would not be an excess of one relative to the other to skew the population of molecules produced by the F plasmid. However pSQ1159 increased the mating frequency of the complemented F plasmid to 76% of that seen with M176. Autoradiograms of probed Northern blots of total RNA isolated from this strain showed that there was a large amount of the *traJ'* RNA present, but no FinP greater than background. Therefore the molecular excess of *traJ'* probably hybridized with the FinP transcribed from the F plasmid, allowing more efficient *traJ* RNA expression from the resident F plasmid. The increased derepression of M176/pED104 cells containing pSQ350 and 351 correlated with a greater amount of *traJ'* RNA detectable on Northern blots. This result too,was consistent with the idea that *traJ'*, and not *traM* transcripts were interacting with the FinOP system.

The restoration of some repression in the totally derepressed strain SL20/pED104/pSQ351 by inducing the transcription of *finP* from the *tac* promoter

confirmed that the interacting molecules were FinP and *traJ'* RNA. Also, comparing the repression levels between SL20/pED104/ pSQ351 and SL20/pED104/pSQ150 showed that F plasmid transfer is derepressed only when the traJ' transcript is produced. Therefore these induction experiments provided strong support for the idea that *traJ* RNA interacts with FinP.

pSQ180 contained only the *finP* gene, and it increased repression 30-fold over M176/pED104 cells, to 0.03% transfer frequency of M176. Therefore increasing the amount of FinP in a cell increases the efficiency of FinOP interaction with *traJ* mRNA. Finally, pSQ150 did not alter the repression of M176/pED104 cells that contained this construct. This was consistent with expectations since it had no promoters for either gene and could not produce either transcript. It served as a control to show that the presence of a multicopy plasmid bearing a fragment of F plasmid DNA does not influence the mating ability of M176.

It was concluded from these experiments that *traM* RNA does not interact with *finP* RNA in a significant manner, and the *traJ'* transcript or the act of its production interferes with the ability of FinOP to repress expression of F plasmid *traJ*.

Chapter VI. Conclusions

This final chapter will attempt to synthesize a model of FinOP and FinOP:*traJ* RNA interactions, using the data presented in this thesis. The data presented will also be compared to that collected from other, better characterized antisense RNA systems to draw conclusions about the molecular interactions involved in the repression of transfer of F-like plasmids.

The data presented support the notion that FinP is an antisense RNA molecule whose abundance depends on its stability and whose efficiency depends, partly, on its abundance. Fig.IV.3 showed that FinP molecules with destabilizing mutations in their stems were less abundant, while FinP with a stabilizing mutation in its stem (pNY300B) was more abundant than wild-type FinP molecules. Experiments described in Chapter V suggest that the amount of FinP unpaired with *traJ* RNA available in a cell is directly related to its ability to repress transfer.

The nature of FinO was addressed in the experiments presented in Chapter IV. It cannot be an antisense RNA molecule complementary to *traJ* RNA, since its stabilizing effect on FinP is noted in the absence of *traJ* RNA. Similarly, it cannot be a protein that, like Rom, stablilizes the initial interactions between the antisense RNA and its target RNA, for its effect on FinP is observed even when there is no *traJ* RNA in the cell, and hence no

opportunity for duplex formation betweeen FinP and *traJ* RNA. It does not function as an inhibitor of a FinP-specific RNase, for if it did so then all the induced RNA produced in the pSQ150 induction experiments in Chapter IV would be protected, and in fact only about 10-20% of it is (fig IV.6, data not shown). The role of FinO that is most consistent with the experimental results presented is that it is a FinP-binding protein that through binding FinP increases the stability of the RNA molecule.

The foregoing considerations have led to the conclusion that FinOP is a protein:RNA complex that acts as an RNP to inhibit the expression of *traJ*.

But what is the nature of the FinOP repression of *traJ*? There are many models to consider. From the mating assay results with M176/pED104/pSQ150 cells, which contained a clone of the target site of FinP, but no active *traJ* promoter, we can conclude that FinOP does not bind to DNA. If FinOP did bind to DNA, this clone would derepress the M176/pED104 host cells, because FinOP would bind to DNA sequences in pSQ150 that produce no *traJ* RNA, thus allowing F plasmid *traJ* DNA to be transcribed.

FinOP does not engage *traJ* RNA in a stable duplex. If it did, this duplex would be observed in M176/pED104 cells, and no *traJ* RNA molecules of a size approximating FinP were observed. And we know that detecting the truncated *traJ* RNA is not difficult, because the *traJ'* complement of FinP is easily detectable and therefore presumably stable. FinOP does not enter into a suicide complex with *traJ* RNA as does OOP RNA with its target transcript (Krinke, Wulff, 1987). In the latter example, hybridization of OOP RNA to the target RNA induces a susceptibility to nuclease degradation of both molecules. In the case of the FinOP system, the concentration of FinP remains constant, while *traJ* transcription is repressed by the FinOP complex. If FinP were tying up *traJ* RNA in a nuclease-susceptible complex, then the induction of *traJ* would result in the reduction of FinP, but it does not. FinOP does not act in a strictly catalytic fashion, for its activity is titratable by increasing the amount of *traJ'* in a cell, as happens with pSQ350 in M176/pED104 cells.

It is clear that FinOP complexes repress at the transcriptional level, for if they acted post-transcriptionally, we would expect to see some molecular remnants of *traJ* mRNA, for reasons discussed above. It is also probable that FinOP does not act at the pre-initiation of transcription stage, or pSQ150 would derepress M176/pED104 cells. It is proposed that the primary method of FinOP repression may be through a transient interaction with RNA polymerase and an elongating *traJ* RNA to repress the transcription of *traJ*. This would explain the apparent independence of the amounts of the two RNA molecules in terms of each other, for if they were interacting as they are in strains like M176/pED104/pSQ350, one would guess that the cells would not contain large amounts of both transcripts - something should be happening to one or both of them. This type of model also explains why FinOP can shut off *traJ* expression, and no *traJ'* RNA is detected.

The most direct way to test this hypothesis would be through *in vitro* transcription experiments. FinO has been successfully overexpressed in our laboratory, and will soon be purified (T. vanBiesen, pers. comm.). Transcription of the *Hpa*II - *Bgl*II fragment that is cloned into pSQ351 in the presence or absense of purified FinP and FinO preparations should allow a direct discrimination between the model presented and a post-transcriptional paradigm.

Chapter VII. Bibliography

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